Interview Summary	Application No. 09/481,572	Applicant(s) SHAN ET AL	
	Examiner Michael V. Meller	Art Unit 1654	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Michael V. Meller*. (3) *Monica Chin Kitts*.

(2) *Steve Giovannetti*. (4)____.

Date of Interview: *02 March 2005*.

Type: a)☐ Telephonic b)☐ Video Conference
c)☒ Personal [copy given to: 1)☐ applicant 2)☒ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☒ No.
If Yes, brief description: ____.

Claim(s) discussed: *All of record*.

Identification of prior art discussed: *All of record*.

Agreement with respect to the claims f)☐ was reached. g)☒ was not reached. h)☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *Examiner explained his rejections and how the art meets the claims. The examiner will mail out a new office action restarting the response period and will withdrawn the restriction requirement*.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04) If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

Examiner's signature, if required

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 7[illegible]4, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-[illegible]face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner wa[illegible]reached at the interview.

Title 37 C[illegible]de of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)
In every instance where reconsideration is requested in view o[illegible]r interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An in[illegible]terview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be tra[illegible]nsacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent an[illegible]Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relati[illegible]n to which there is disagreement or doubt.

The action of the Patent and Trademark Offi[illegible]e cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance [illegible]f interviews.

It is the responsibility of the applicant or the a[illegible]ttorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the ex[illegible]aminer's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Sum[illegible]mary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling [illegible]in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwi[illegible]se provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office a[illegible]ctions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an [illegible]Examiner's Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given [illegible]an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal int[illegible]erview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone [illegible]or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If [illegible]additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed prom[illegible]ptly after the interview rather than with the next official communication.

The Form provides for recordation of the follo[illegible]wing information:
- Application Number (Series Code and Se[illegible]rial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-confe[illegible]rence, or personal)
- Name of participant(s) (applicant, attorne[illegible]y or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit w[illegible]as shown or a demonstration conducted
- An identification of the specific prior art di[illegible]scussed
- An indication whether an agreement was [illegible]reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or c[illegible]laims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner [illegible]to the contrary.
- The signature of the examiner who condu[illegible]cted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind [illegible]the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary [illegible]Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant [illegible]or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the sub[illegible]stance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exh[illegible]ibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art disc[illegible]ussed,
4) an identification of the principal proposed a[illegible]mendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the [illegible]Examiner,
5) a brief identification of the general thrust of [illegible]the principal arguments presented to the examiner,
   (The identification of arguments need [illegible]not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arg[illegible]uments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the co[illegible]ntext of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he o[illegible]r she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent [illegible]matters discussed, and
7) if appropriate, the general results or outcom[illegible]e of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review th[illegible]e applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extend[illegible]able one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons o[illegible]n record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is comp[illegible]lete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along wit[illegible]h the date and the examiner's initials.

Cardiovascular care with the new T-type calcium channel antagonist: possible role of attendant sympathetic nervous system inhibition

Fritz R. Bühler

Differences among types of calcium antagonists Calcium antagonists lower blood pressure, relieve angina pectoris and improve chronic heart failure, primarily through peripheral and coronary vasodilation. The debate as to whether short-acting, long-lasting (L)-channel calcium influx antagonists of the 1,4-dihydropyridine type might be involved in excess cardiac mortality has raised new controversies with respect to the cardiac morbidity and mortality outcome for all calcium antagonists. Different pharmacodynamic effects (short-acting vasodilation inducing pulsatile sympathetic reflex stimulation) may explain differences in outcome with calcium antagonist therapies. Calcium antagonists also differ in their direct effects on the sympathetic nervous system, and on its long endocrine arm, the renin–angiotensin–aldosterone system. These differential effects relate to the cardiac conduction system and ventricular ectopic activity, to cardiac and vascular remodelling and hypertrophy, and perhaps also to the development of hypertension.

L-channel calcium antagonists Depending on their pharmacodynamic characteristics, L-channel calcium antagonists of the dihydropyridine, verapamil or diltiazem type reflexly activate the sympathetic nervous system and blunt β-adrenoceptor-mediated calcium influx, thus eliciting negative inotropy and activation of the renin–angiotensin system. Both verapamil and diltiazem slow down pacemaker activity and atrioventricular conduction.

Mibefradil The new-class T-channel blocker mibefradil exhibits vascular selectivity and induces peripheral and coronary vasodilation. There is no reflex sympathetic activation and no negative inotropic effect. It increases coronary blood flow without increasing oxygen consumption and causes a slight slowing of the heart rate, thereby inducing diastolic relaxation. The latter improves subendocardial and small artery perfusion. There is a sympatholytic effect, owing to T-channel expression in neurones, sinoatrial and atrioventricular nodes and Purkinje fibres. In experimental models, ventricular ectopic activity is reduced with mibefradil. The renin–angiotensin–aldosterone system and endothelin effects are blunted by T-channel inhibition. These and other factors reduce smooth muscle cell proliferation, hypertrophy and matrix deposition. T-type calcium channel inhibition, over and above its antihypertensive and anti-ischaemic effects, and afterload-reducing effects in chronic heart failure, offers the potential for a cardiovascular protective benefit, which may be critically related to interference with the sympathetic nervous system.

Journal of Hypertension 1997, 15 (suppl 5):S3–S7

Keywords: calcium T-channel antagonist, sympathetic system, cardiovascular care

From the Division of Cardiology and European Center of Pharmaceutical Medicine, University Hospital, Basel, Switzerland.

Requests for reprints to Prof. Fritz R. Bühler, ECPM, University Hospital, CH-4031 Basel, Switzerland.

 ISSN 0952–1179

Introduction

In the last 30 years all calcium antagonists used in clinical practice have been more or less hampered by their negative inotropic effect. The 1,4-dihydropyridines (nifedipine as a prototype) evoke increases in the heart rate, reflecting reactive increases in sympathetic nerve activity. The phenylalkylamines (verapamil) and the benzodiazepines (diltiazem) have direct negative chrono- and dromotropic effects that may mask reflex sympathetic activation.

Cardiovascular pharmacotherapy with attendant slowing of the heart is associated with reduced cardiac morbidity and mortality [1–3]. This is supported by the abundance of information on the cardioprotective effects of β-blockers [4] and angiotensin converting enzyme (ACE) inhibitors [5]. However, mechanisms other than heart rate slowing are also operative. Interference with the sympathetic nervous system at several prejunctional and adrenoceptor levels is an inherent mechanism of β-blockers, ACE inhibitors and angiotensin antagonists [6].

In recent years, an ardent debate has arisen over a potentially adverse effect of calcium antagonists, in particular the short-acting, L-type (long-lasting) channel-blocking dihydropyridines, which have been accused of causing excess cardiac mortality, especially in postinfarction trials [7,8]. The data that have been presented so far, however, are insufficient and the conclusions drawn from meta-analyses can best be used as hypothesis-generating; they do not imply a necessary change in treatment strategies. A series of ongoing prospective trials

will clarify this situation [9]. Knowledge of how calcium antagonists directly and reflexly interact with the sympathetic nervous system may help to explain differential cardiac protective observations. These effects of calcium antagonists are basically threefold: pharmacodynamic effects (short-acting vasodilation inducing pulsatile sympathetic reflex stimulation), direct cardiovascular peripheral effects (for example, pacemaker cells and conduction system) and direct neuronal effects. The new-class calcium antagonist mibefradil offers sympatholytic and antiproliferative effects, and should thereby be viewed as a potentially cardiovascular-protective agent.

Therapeutic gain in selectivity from L to T calcium channel inhibition

In the fine-tuning of cardiovascular pharmacotherapy with calcium antagonists (of the L-channel-blocking type), optimal use of these drugs may have been hampered by four important shortcomings: a lack of smooth onset and 24-h duration of action, a negative inotropic effect, sympathetic counter-regulation following vasodilation and an alleged lack of long-term cardiovascular protective effects. Optimization of pharmacokinetic and pharmacodynamic profiles of classical calcium antagonists [10] has clearly been achieved by both galenical improvements and the development of a new generation of long-acting L-channel inhibitors such as amlodipine and felodipine.

Various calcium antagonists have proved to be partially selective for vascular smooth muscle, with depressant actions on heart contraction limited to less than 50% of that on vascular muscle when measured as a percentage of force reduction [11,12]. Mibefradil (1 μmol/l) partially (about 25–30%) blocks L channels, but blocks 95% of T-type (transient) calcium channel currents and smooth muscle contraction [13]. Therefore, mibefradil has clearly demonstrated T-type calcium channel selectivity. The use of this new class of calcium antagonist has shown that a predominant T-channel block produces effective and smooth peripheral vasodilation without cardiac depression. In contrast, amlodipine, felodipine, nitrendipine, nisoldipine, nimodipine, isradipine, diltiazem and verapamil are all selective blockers of L-type channels with only minor, if any, effects on T-type calcium channels at therapeutically relevant concentrations [13].

Part of the highly mitigated sympathetic counter-regulation following powerful vasodilation may be due to the 'strategic' distribution of T channels relative to the therapeutic needs. Effects related to T channels in the sinus node, atrioventricular node and ventricular conduction system are relatively easy to demonstrate. T channels also seem to have a stimulatory role in central and peripheral nervous transmission [14], though mibefradil does not seem to cross the blood–brain barrier. In contrast to T channels, L channels are not expressed in the nervous system. The pattern of expression of T channels along the cardiovascular tree as well as in neurones may explain the unique effectiveness of T-type calcium channel antagonists for clinical use.

T-channel inhibition and cardiac and vascular responses to injury

A sympatholytic effect may combine with a long-term antiproliferative and remodelling effect in the cardiovascular system to increase cardiovascular protection. Both T channels and L channels are overexpressed in cardiac hypertrophy [15,16], itself a powerful risk factor [17]. Reduction of left ventricular hypertrophy with the L-channel inhibitors has been well documented [18], but this has not been as marked as that clearly demonstrated with ACE inhibitors [19] or as high as the level so far reported via T-channel inhibition with mibefradil [20]. It is conceivable that the T channel may be a key factor in the control of the growth of cardiac and vascular smooth muscle cells.

There is ample information on L-channel calcium antagonist effects on remodelling and the response to injury following endothelial denudation with carotid ballooning (neointimal formation and early development of atherosclerosis) [21]. Mibefradil has been reported to attenuate the neointimal formation in response to ballooning by at least 50% [22]. Immunohistochemical analysis of proliferating cell nuclear antigen revealed that mibefradil markedly curtailed both the onset and the extent of neointimal cell replication [22]. A recent study indicated that although mibefradil did not affect synthesis/turnover of the extracellular matrix glycoproteins fibronectin, laminin and collagens (types I, III and IV), it exerted marked inhibitory effects on expression of the glycoprotein tenascin. In the placebo group of ballooned animals, tenascin expression coincided with medial smooth muscle cell migration–replication and neointimal formation. Thus, mibefradil appears to be effective at both early and late stages in the remodelling process (T.J. Resink, F. Kern, A.W.A. Hahn, F.R. Bühler, R. Schmitt, manuscript submitted for publication). It is also known that the T-type calcium influx mediates the sustained intracellular calcium increase induced by angiotensin II and platelet-derived growth factor [23,24]. Thus the T channel appears to be a common denominator of growth control.

Although practically all atherogenic factors are calcium-sensitive with the different calcium antagonists [23], little if any effect has been shown on human atherosclerosis. In the coronary system, only the early onset and development of atherosclerotic plaques was shown to have been blunted by nifedipine (International Nifedipine Trial on Antiatherosclerotic Therapy, INTACT [24]). The Multicenter Isradipine/Diuretic Atherosclerosis Study (MIDAS) using Doppler ultrasound detection of plaques in the carotid artery was negative for the calcium antagonist [25]. There is still no evidence that L-type calcium channel inhibition has a relevant impact on the development of atherosclerosis in man. The apparent reductive effects of T-channel inhibition on cell migration, proliferation and matrix formation and neuro-endocrine transmission in experimental models may indicate therapeutic potential in vascular remodelling. This cardiovascular remodelling effect and the anti-atherosclerotic potential of the new-

class T-channel antagonist mibefradil still requires evaluation in the clinical setting.

Predictable and smooth antihypertensive and anti-anginal therapy

In recent years, L-type calcium channel antagonists have been engineered for a smooth onset and 24-h action with optimized galenical formulations, or with the development of the third generation of L-channel inhibitors which inherently exhibit these characteristics. While short-acting calcium antagonists clearly cause repetitive sympathetic reflex activation [26], evidence is accumulating that smoother acting L-channel inhibitors may also evoke increases in (sural) sympathetic nerve activity [27], as also reflected in increased plasma catecholamine and renin responses, with both acute and chronic treatment [26,28–30].

Mibefradil has greater than 90% bioavailability, a slow rate of elimination, and produces a high trough to peak ratio and thus a clear and smooth 24-h antihypertensive action. This optimal kinetic and dynamic profile suggests a high predictability of response for use in clinical practice [31]. Thus both antihypertensive and anti-anginal effectiveness may be obtained with this T-type calcium antagonist. So far, the anti-anginal effects of L-type calcium channel antagonists have not been overly impressive, perhaps due to a reflex increase in sympathetic activity. A lack of this reflex may contribute to and explain the anti-anginal results reported so far for mibefradil [32].

The cardiac morbidity–mortality outcome debate

Although calcium antagonists are widely used in the treatment of cardiac ischaemia and angina, hypertension and, to some extent, heart failure, recent meta-analyses of a variety of trials have linked some calcium antagonists, mainly of the short-acting dihydropyridine type, to excess cardiac events [33]. In addition, two case–control studies published in 1995 raised concerns that the treatment of hypertensive patients with calcium antagonists might increase the risk of myocardial infarction [34,35]. This was followed by an extension of other meta-analyses, demonstrating that high doses of short-acting nifedipine capsules increase the risk of recurrent myocardial infarction [33,36]. Since that time, there have been extensive debates in the literature and at scientific meetings. Final resolution of this issue will not be possible until complete publication of long-term, prospective, randomized, outcome studies of morbidity and mortality. Most recently, the Systolic blood pressure in Europe (Syst-Eur) study showed reduced cardiac mortality with nitrendipine [37]. In prospective collaborative overviews of major ongoing randomized trials of antihypertensive treatments, the newly established World Health Organization–International Society of Hypertension (WHO–ISH) Pressure Lowering Treatment Trialists' Collaboration (PLTTC) plans to report on meta-analyses of 75 000 patients (about 30 000 treated with calcium antagonists) in 15 trials in 1999 and of a total of 197 000 (about 105 000 treated with calcium antagonists) in 2003 [9]. These prospective and well powered trials are required in order to provide benchmarks of the effects of β-blockers (and diuretics) on myocardial infarction and stroke, and of ACE inhibitors on mortality in heart failure patients.

In the meantime, further mechanistic clarification of sympathetic counter-regulation following peripheral vasodilation is needed. One current hypothesis is that repetitive acute vasodilation by shorter-acting L-channel type calcium antagonists might have adverse effects resulting from repeated reflex sympathetic stimulation. Accordingly, antihypertensive effects could be blunted and angina could be induced. Proposed regression of left ventricular hypertrophy is impaired by repeat sympathetic activation, as demonstrated with the use of felodipine twice a day [38]. The combination of sympathetic activation and reduced coronary perfusion, as blood pressure falls, may explain the ischaemic complications of short-acting L-channel inhibitors [39]. Concomitant therapy with ACE inhibition buffers the regulatory response [40], as does β-blocker therapy [41]. Nifedipine is more likely to increase catecholamines and renin activity than the slower-onset calcium antagonist verapamil [42]. In chronic use of L-channel inhibitors, there is some masked reflex sympathetic activation, as it can be uncovered by the addition of a β-blocker to nifedipine therapy [41].

The possible effect of a fall in the heart rate has been demonstrated by the administration of verapamil and diltiazem to patients after a myocardial infarction, although here, not unexpectedly, benefit was shown only in patients without heart failure or pulmonary congestion [43,44].

Against this background, the new class of T-channel calcium antagonists, with their reduced likelihood for inducing adverse events of reflex sympathetic nerve activation and negative inotropy, may provide a new dimension in cardiovascular care.

Is neurohormonal reflex activation the problem?

There is very clear evidence that the acute administration of calcium antagonists causes reflex increases in heart rate, plasma catecholamines and plasma renin activity [26], and there is a direct increase in sural sympathetic nerve activity supplying skin or skeletal muscle [27]. Sural nerve activity is a directly measurable effect on sympathetic nerve activity which is independent of the direct slowing effects on pacemaker cells and atrioventricular conduction. More recently, the differential effects of the 1,4-dihydropyridines verapamil and diltiazem have been tested in the haemodynamic system, and not only acute but also chronic administration of calcium antagonists led to a sustained elevation of nerve activity in the following order: short-acting dihydropyridines > longer-acting dihydropyridines > diltiazem > verapamil [45].

T-type calcium channel inhibition with mibefradil seems to be associated with a smooth overall reaction of the sympathetic nerve system, since there appears to be no effect on catecholamines and renin and no effect on reactive sural nerve activity, particularly in view of powerful vasodilation. It is therefore conceivable that in addition to a beneficial slowing of the heart rate, mibefradil exerts a peripheral inhibition of nerve transmission. The evidence also suggests that aldosterone stimulation by angiotensin is mediated by T-type channels [46] and that renin release is induced [47]. Thus, the renin–angiotensin system, the long endocrine arm of the sympathetic nervous system, is blunted.

These differential effects on reflex sympathetic activation could explain why some calcium antagonists have little effect on cardiovascular morbidity and mortality, while others such as diltiazem and verapamil seem to be beneficial (as long as sympathetic reactivity is not increased by severe heart failure).

Moreover, there is experimental evidence that mibefradil greatly reduces ventricular tachycardia and fibrillation following postischaemic reperfusion [48] and prolongs survival following myocardial infarction in the rat [49], thus pointing to its potential for reducing sudden death in man.

Calcium antagonists and heart failure

Congestive heart failure remains an important cause of death and disability with annual mortality rates of 10–50%, depending on the severity [50]. Improvement of the haemodynamic abnormalities does not of itself lead to lower risk [51], suggesting additional pathogenic factors. Disease progression has been related to neurohormonal [52] and angiotensin system activation [53].

Because of the negative inotropic effect of all L-calcium channel antagonists, their use in heart failure is limited. Their therapeutic risk appears to be dependent upon the ratio between the degree of negative inotropy and the degree of compensatory reflex sympathetic counteractivation. The latter helps to overcome the direct negative inotropic effects of L-channel inhibition. Both effects develop more smoothly with third-generation long-acting dihydropyridines such as amlodipine. In the Prospective Randomized Amlodipine Survival Evaluation (PRAISE) study in heart failure patients, no harmful effects have been observed [54].

The new-class T-type calcium channel blocker mibefradil may have a special role in heart failure therapy because of its pharmacokinetic and pharmacodynamic characteristics. Its smooth onset and long duration of action, afterload-reducing effects, heart rate-slowing and sympatholytic actions may add a new dimension to heart failure patients who have elevated sympathetic nervous system activity. A calcium antagonist such as mibefradil may thus provide at least comparable benefits to those observed with ACE inhibitors [55], the angiotensin II antagonist losartan [56] or a vasodilator with a β-adrenoceptor-blocking component [57].

A large mortality study with mibefradil, the first Mortality Assessment in Congestive Heart failure patients (MACH-1) [58], is under way at present and may clarify these T-channel-related issues. A total of 2600 patients have been recruited for this 2.5-year treatment trial; results are expected in 1998. On the basis of an interim analysis, the Independent Safety Committee recently recommended the continuation of the study. In addition to assessing the long-term safety and efficacy of mibefradil therapy in patients with heart failure, the study may reveal effects on morbidity, quality of life and sudden cardiac death in these patients.

References

1 Kannel WB, Kannel C, Paffenbarger RS Jr, Cupples LA: Heart rate and cardiovascular mortality: the Framingham Study. *Am Heart J* 1987, 113:1489–1494.
2 Palatini P, Julius S: Heart rate and cardiovascular risk. *J Hypertens* 1997, 15:3–17.
3 Habib G: Reappraisal of the importance of heart rate as a risk factor for cardiovascular morbidity and mortality. *Clin Ther* 1997, 19:39–52.
4 Yusuf S, Peto R, Lewis J, Collins R, Sleight P: Beta-blockade during and after myocardial infarction: an overview of the randomized trials. *Prog Cardiovasc Dis* 1985, 27:335–371.
5 Lonn E, Yusuf S, Prabhat J, *et al.*: Emergency role of angiotensin-converting enzyme inhibitors in cardiac and vascular protection. *Circulation* 1995, 92:1328–1331.
6 Bühler FR: Antihypertensive care with calcium antagonists. In *Hypertension, Pathophysiology, Diagnosis and Management, 2nd edn.* Edited by Laragh JH, Brenner BM. New York: Raven Press; 1995:2801–2814.
7 Yusuf S, Held P, Furberg C: Update of effects of calcium antagonists in myocardial infarction or angina in light of the second Danish Verapamil Infarction Trial (DAVIT II) and other recent studies. *Am J Cardiol* 1991, 67:1295–1297.
8 Opie LH, Messerli FH: Nifedipine and mortality: grave defects in the dossier. *Circulation* 1995, 92:1068–1073.
9 World Health Organization–International Society of Hypertension Blood Pressure Lowering Treatment Trialists' Collaboration: Protocol for prospective collaborative overviews of major randomized trials of blood pressure lowering treatments. *J Hypertens* (in press).
10 Opie LH, Bühler FR, Fleckenstein A, *et al.*: International Society and Federation of Cardiology: Working Group on Classification of Calcium Antagonists for Cardiovascular Disease. *Am J Cardiol* 1987, 60:630–632.
11 Clozel JP, Banken L, Osterrieder W: Effects of Ro 40-5967, a novel calcium antagonist, on myocardial function during ischaemia induced by lowering coronary perfusion pressure in dogs: comparison with verapamil. *J Cardiovasc Pharmacol* 1989, 14:713–721.
12 Veniant M, Clozel JP, Hess P, Wolfgang R: Haemodynamic profile of Ro 40-5967 in conscious rats: comparison with diltiazem, verapamil, and amlodipine. *J Cardiovasc Pharmacol* 1991, 18 (suppl 19):S55–S58.
13 Mishra SK, Hermsmeyer K: Selective inhibition of T-type Ca^{2+} channels by Ro 40-5967. *Circ Res* 1994, 75:144–148.
14 Catterall WA, Striessnig J: Receptor sites for Ca^{2+} channel antagonists. *Trends Pharmacol Sci* 1992, 131:256–262.
15 Sen L, Smith T: T-type Ca^{2+} channels are abnormal in genetically determined cardiomyopathic hamster hearts. *Circ Res* 1994, 75:149–155.
16 Nuss H, Houser S: T-type Ca^{2+} current is expressed in hypertrophied adult feline left ventricular myocytes. *Circ Res* 1993, 73:777–782.
17 Kannel WB, Gordon T, Lastelli WP, *et al.*: Electrographic left ventricular hypertrophy and risk of coronary heart disease: the Framingham Study. *Ann Intern Med* 1970, 72:813–825.
18 Schmieder RE, Martus P, Klingbeil A: Reversal of left ventricular hypertrophy in essential hypertension. *JAMA* 1996, 275:1507–1513.
19 Dahlöf B, Pennert K, Hansson L: Reversal of left ventricular hypertrophy in hypertensive patients: a meta-analysis of 109 treatment studies. *Am J Hypertens* 1992, 5:95–110.

20 Höglund C, Pardy R, Watt A, *et al.*: A comparison of the effects of mibefradil and atenolol on left ventricular hypertrophy regression in hypertensive patients. *Am J Cardiol* (in press).

21 Catapano AL: Calcium antagonists and atherosclerosis: experimental evidence. *Eur Heart J* 1997, 18 (suppl A):A80–A86.

22 Schmitt R, Clozel J-P, Iberg N, Bühler FR: Mibefradil prevents neointimal formation after vascular injury in rats: possible role of the blockade of the T-type voltage operated calcium channel. *Arterioscler Thromb Vasc Biol* 1995, 15:1161–1165.

23 Hahn AWA, Kern F, Jonas U, John M, Bühler FR, Resink TJ: Functional aspects of vascular tenascin-C expression. *J Vasc Res* 1995, 32:152–174.

24 Lafleur DE, Fagin JA, Forrester S, Rubin SA, Sharifi BG: Cloning and characterisation of alternatively spliced isoforms of rat tenascin: platelet-derived growth factor-BB markedly stimulates expression of spliced variants of tenascin mRNA in arterial smooth muscle cells. *J Biol Chem* 1994, 269:20757–20763.

25 Borhani NO, Mercuri M, Borhani PA, *et al.*: Final outcome results of the Multicenter Isradipine Diuretic Atherosclerosis Study (MIDAS): a randomized controlled trial. *JAMA* 1996, 276:785–791.

26 Kiowski W, Erne P, Bertel O, Bolli P, Bühler FR: Acute and chronic sympathetic reflex activation and antihypertensive response to nifedipine. *J Am Coll Cardiol* 1986, 7:344–348.

27 Noll G, Wenzel RR, Allegranza G, Lüscher TF: Nifedipine activates sympathetic nervous system: additive effects of cold pressor test [abstract]. *Eur Heart J* 1994, 15 (suppl 15):190.

28 Ruzicka M, Leenen FHH: Relevance of intermittent increases in sympathetic activity for adverse outcome on short-acting antagonists. In *Hypertension, Diagnosis, and Management, 2nd edn.* Edited by Laragh JH, Brenner BM. New York: Raven Press; 1995:2815–2825.

29 Schmitt R, Kleinbloesem C, Belz G, *et al.*: Haemodynamic and humoral effects of the novel calcium antagonist Ro 40-5967 in patients with hypertension. *Clin Pharmacol Ther* 1992, 52:314–323.

30 Wagner C, Krämer B, Kurtz A: T-type and L-type calcium channel blockers exert opposite effects on renin secretion and renin gene expression in conscious rats. *Br J Pharmacol* (in press).

31 Bernink P, Prager G, Schelling A, Kobrin I: Antihypertensive properties of the novel calcium antagonist mibefradil (Ro 40-5967): a new generation of calcium antagonists? *Hypertension* 1996, 27:426–432.

32 Braun S, van der Wall E, Emanuelsson H, Kobrin I: Effects of a new calcium antagonist, mibefradil (Ro 40-5967), on silent ischaemia in patients with stable chronic angina pectoris: a multicenter placebo-controlled study. *J Am Coll Cardiol* 1996, 28:317–322.

33 Furberg CD, Psaty BM, Meyer JV: Nifedipine dose-related increase in mortality in patients with coronary heart disease. *Circulation* 1995, 92:1326–1331.

34 Psaty BM, Heckbert SR, Koepsell TD, *et al.*: The risk of myocardial infarction associated with antihypertensive drug therapies. *JAMA* 1995, 274:620–625.

35 Pahor M, Guralnik JM, Corti M, *et al.*: Long-term survival and use of antihypertensive medication in older persons. *J Am Geriat Soc* 1995, 43:1191–1197.

36 Alderman MH, Cohen H, Roque R, Madhavan S: Effect of long-acting and short-acting calcium antagonists on cardiovascular outcomes in hypertensive patients. *Lancet* 1997, 349:594–598.

37 Staessen JA, Fagard R, Thijs L, *et al.*: Randomised double-blind comparison of placebo and active treatment for older patients with isolated systolic hypertension: the Systolic hypertension in Europe (Syst-Eur) trial investigators. *Lancet* 1997, 350:757–763.

38 Leenen FHH, Holliwell DL: Antihypertensive effect of felodipine associated with persistent sympathetic activation and minimal regression of left ventricular hypertrophy. *Am J Cardiol* 1992, 69:639–64S.

39 Ruzicka M, Leenen FHH: Relevance of 24 h blood pressure profile and sympathetic activity for outcome on short- versus long-acting 1,4-dihydropyridines. *Am J Hypertens* 1996, 9:86–94.

40 Bellet M, Sassano T, Guyenne P, *et al.*: Converting-enzyme inhibition buffers the counter-regulatory response to acute administration of nicardipine. *Br J Clin Pharmacol* 1997, 24:465–472.

41 Redfield MM, Neumann A, Tajik AJ, *et al.*: Effect of long-acting oral felodipine and nifedipine on left ventricular contractility in patients with systemic hypertension: differentiation between reflex sympathetic and direct contractile responses [abstract]. *J Am Coll Cardiol* 1994, 23:348A.

42 Muiesan G, Agabiti-Rosei E, Romanelli G, *et al.*: Adrenergic activity and left ventricular function during treatment of essential hypertension with calcium antagonists. *Am J Cardiol* 1988, 57:44D–49D.

43 The Danish Study Group on Verapamil in Myocardial Infarction: Effect of verapamil on mortality and major events after acute myocardial infarction: the Danish Verapamil Infarction Trial II (DAVIT-II). *Am J Cardiol* 1990, 66:779–785.

44 The Multicenter Diltiazem Postinfarction Trial Research Group: The effect of diltiazem on mortality and reinfarction after myocardial infarction. *N Engl J Med* 1988, 319:385–392.

45 Wenzel RR, Allegranza G, Binkert C, *et al.*: Differential activation of cardiac and peripheral nervous system by nifedipine: role of pharmacokinetics. *J Am Coll Cardiol* 1997, 29:1207–1214.

46 Buisson B, Laflamme L, Bottari S, *et al.*: A G protein is involved in the angiotensin AT_2 receptor inhibition of the T-type calcium current in non-differential NG108-15 cells. *J Biol Chem* 1995, 270:1670–1674.

47 DeGasparo M, Bottari S, Levens N: Characteristics of angiotensin II receptors and their role in cell and organ physiology. In *Hypertension: Pathology, Diagnosis, and Management, 2nd edn.* Edited by Laragh JH, Brenner BM. New York: Raven Press; 1995:1695–1720.

48 Billmann GE, Hamlin RL: The effects of mibefradil, a novel calcium channel antagonist, on ventricular arrhythmias induced by myocardial ischemia and programmed electrical stimulation. *J Pharmacol Exp Ther* 1996, 277:1517–1526.

49 Mulder P, Richard V, Compagnon P, *et al.*: Increased survival after long-term treatment with mibefradil, a selective T-channel calcium antagonist, in heart failure. *J Am Coll Cardiol* 1997, 29:416–421.

50 Massie B: Is neurohormonal activation deleterious to the long-term outcome of patients with congestive heart failure? *J Am Coll Cardiol* 1988, 12:547–548.

51 Packer M: The neurohormonal hypothesis: a theory to explain the mechanism of disease progression in heart failure. *J Am Coll Cardiol* 1992, 20:248–254.

52 Cohn J, Levine T, Olivari M, *et al.*: Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. *N Engl J Med* 1984, 311:819–823.

53 Laragh JH: Renin–angiotensin–aldosterone system for blood pressure and electrolyte homeostasis and its involvement in hypertension, in congestive heart failure and in associated cardiovascular damage (myocardial infarction and stroke). *J Hum Hypertens* 1995, 9:385–390.

54 Packer M, O'Connor CM, Ghali JK, *et al.*: Effect of amlodipine on morbidity and mortality in severe chronic heart failure: Prospective Randomized Amlodipine Survival Evaluation Study Group. *N Engl J Med* 1996, 335:1107–14.

55 The CONSENSUS Trial Study Group: Effects of enalapril on mortality in severe congestive heart failure: results of the Cooperative North Scandinavian Enalapril Survival Study. *N Engl J Med* 1987, 316:1429–1435.

56 Pitt B, Segal R, Martinez F, *et al.*: Randomised trial of losartan versus captopril in patients over 65 with heart failure (Evaluation of Losartan in the Elderly Study, ELITE). *Lancet* 1997, 349:747–752.

57 Packer M, Bristow M, Cohn J, *et al.*: The effect of carvedilol on morbidity and mortality in patients with chronic heart failure: US Carvedilol Heart Failure Study Group. *N Engl J Med* 1996, 334:1349–1355.

58 Levine TB: The design of the Mortality Assessment in Congestive Heart Failure Trial (MACH-1, mibefradil). *Clin Cardiol* 1997, 20:320–326.

Journal of Cardiovascular Pharmacology™
29:692–696 © 1997 Lippincott–Raven Publishers, Philadelphia

Effects of the Novel T-Type Calcium Channel Antagonist Mibefradil on Human Myocardial Contractility in Comparison with Nifedipine and Verapamil

Bodo Cremers, Markus Flesch, *Michael Südkamp, and Michael Böhm

*Klinik III für Innere Medizin der Universität zu Köln; and *Klinik und Poliklinik für Herzchirurgie der Universität zu Köln, Cologne, Germany*

Summary: Mibefradil (Ro 40-5967) is a novel nondihydropyridine calcium antagonist. The aim of our study was to compare the negative inotropic effects of the well-known 1,4-dihydropyridine nifedipine and the phenylalkylamine verapamil with those of mibefradil. Isometric force of contraction in response to these substances was determined in isolated, electrically driven left ventricular papillary muscle strips from failing human hearts (1 Hz, 37°C). The hearts were obtained during cardiac transplantation ($n = 9$) and mitral valve–replacement operations ($n = 9$). The calcium antagonists studied significantly ($p < 0.05$) depressed basal force of contraction in a concentration-dependent manner. The effect started at concentrations >0.001 μM for nifedipine and >0.01 μM for verapamil, but only at concentrations >10 μM for mibefradil. Only in the presence of nifedipine and verapamil was a significant rightward shift of the inotropic concentration–response curves to calcium and a depression of the maximal effects of calcium observed. With respect to the relation between the therapeutic active plasma concentration in vivo and the negative inotropic potency in vitro, it became evident that the difference between therapeutically beneficial concentrations and potentially hazardous cardiodepressant activity increases from nifedipine to mibefradil. We conclude that this new generation of calcium antagonists, almost lacking cardiodepressant effects, could lead to a greater therapeutic index and greater safety in the treatment of cardiovascular diseases. **Key Words:** Mibefradil—Nifedipine—Verapamil—Calcium antagonists—Human myocardium—Heart failure.

Over the past 20-year period, calcium antagonists have been used for the treatment of hypertension and chronic stable angina pectoris. However, a number of recent retrospective analyses have suggested that calcium antagonists may be detrimental and may promote adverse cardiovascular events (1,2). Possible explanations for adverse effects include proischemic effects (3), negative inotropic effects (4), marked hypotension (5), recently reported prohemorrhagic effects (6), and possibly proarrhythmic effects of calcium antagonists (7). For verapamil, similar negative effects have been reported (8,9).

The negative inotropic effects of calcium antagonists in vitro (4) are usually compensated in vivo by a reflex sympathetic stimulation because of the strong peripheral vasodilation induced by these drugs. However, the cardiodepressant effect of calcium antagonists can be detrimental in patients with decreased left ventricular function. In patients with chronic heart failure, baroceptor sensitivity is decreased (10), and adrenergic reflex mechanisms are blunted (7). Therefore the compensatory mechanism might be insufficient to overcome cardiodepression by calcium antagonists in patients with heart failure.

Many efforts are under way to develop second- and third-generation calcium channel blockers with a more favorable pharmacokinetic profile, leading to a higher specificity for vascular tissue and a reduced negative inotropic potency. Mibefradil, a benzimidazolyl-substituted tetraline derivative, is one of these novel calcium antagonists. It belongs to a new chemical class, although it binds to the verapamil-type receptor of cardiac membranes (11). Mibefradil is characterized by its selective and complete blocking properties on the voltage-operated T-type channel, although it has reduced blocking properties on the L-type channel (12).

Our study was designed to compare the negative inotropic effects of mibefradil with those of the well-established calcium antagonists nifedipine and verapamil in vitro by using tissue from failing left ventricular human heart and to relate the results to the therapeutic active plasma concentrations of these compounds. It is

Received August 30, 1996; revision accepted February 10, 1997.
Address correspondence and reprint requests to Prof. M. Böhm at Klinik III für Innere Medizin der Universität zu Köln, Joseph-Stelzmann-Strasse 9, 50924 Köln, Germany.

mandatory to use human left ventricular preparations to overcome possible species differences (13).

In isolated cardiac preparations, beating frequency can be kept constant, and effects of pre- or afterload changes and sympathetic reflex mechanisms do not have to be taken into account.

METHODS

Electrically stimulated human papillary muscle strips

Experiments were performed on isolated, electrically stimulated papillary muscle strips from human left ventricular myocardium. Myocardial ventricular tissue from terminally failing human hearts (NYHA Class IV) was obtained during cardiac transplantation. The preoperative diagnoses were dilated cardiomyopathy (five patients) and ischemic cardiomyopathy (four patients). Tissue from moderately failing human hearts (NYHA Classes II–III) was received during mitral valve-replacement operations (nine patients). All patients gave written informed consent before surgery. The pretreatment of the patients consisted of diuretics, nitrates, angiotensin-converting enzyme inhibitors, and cardiac glycosides. Patients who received catecholamines, β-blockers, or calcium channel antagonists were withdrawn from the study. Cardiac surgery was performed with cardioplegic arrest during hypothermia. The cardioplegic solution was a modified Bretschneider solution containing (in m*M*): NaCl, 15; KCl, 10; $MgCl_2$, 4; histidine HCl, 180; tryptophan, 2; mannitol, 30; and potassium dihydrogen oxoglutarate, 1.

Immediately after explantation, the hearts were placed in ice-cold preaerated (95% O_2/ 5% CO_2) modified Tyrode's solution (for composition, see the following). Left ventricular papillary muscles were split into thin strips of uniform size (diameter, <1 mm; length, 6–8 mm). The preparations were mounted individually in an organ bath containing 75 ml Tyrode's solution (in m*M*): NaCl, 119.8; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 1.05; NaH_2PO_4, 0.42; $NaHCO_3$, 22.6; Na_2EDTA, 0.05; ascorbic acid, 0.28; and glucose, 5.0; maintained at 37°C and aerated continuously with 95% O_2 and 5% CO_2 (pH, 7.4). The muscle strips were paced electrically by two platinum electrodes (frequency, 1 Hz; impulse duration, 5 ms; voltage, 10–20% above threshold). Each muscle strip was stretched to the length at which force of contraction was maximal (~10 mN). Isometric force of contraction was measured with an inductive force transducer (Hottinger Baldwin Messtechnik, Munich, Germany) attached to a Gould recorder (Brush 2400; Gould, Cleveland, OH, U.S.A.).

MATERIALS

Mibefradil (Ro 40-5967) was obtained from Hoffmann-La Roche AG (Basel, Switzerland), nifedipine from Bayer AG (Leverkusen, Germany), and verapamil from Knoll AG (Ludwigshafen, Germany). All other chemicals were of analytic grade or the best grade commercially available.

STATISTICS

Data are expressed as means ± SEM. Statistical significance was analyzed by using Student's *t* test for unpaired observations; $p < 0.05$ was considered significant. The drug concentration producing 50% of the maximal effect (EC_{50} value) was determined in each individual experiment. EC_{50} values are given as means with 95% confidence limits.

RESULTS

Representative original tracings illustrating the inotropic effects of mibefradil and nifedipine in isolated, electrically driven human left ventricular papillary muscle strips are shown in Fig. 1A. It becomes evident that nifedipine had a pronounced negative inotropic effect on the examined papillary muscle strips, whereas mibefradil exerted only a small negative inotropic effect at very high concentrations. The concentration dependency of the negative inotropic effect of nifedipine, but also of verapamil, becomes even more obvious in the cumulative concentration–response curves shown in Fig. 1B. Basal force of contraction was 2.5 (±0.2) mN for nifedipine, 2.8 (±0.8) mN for mibefradil, and 2.4 (±0.3) mN for verapamil, respectively. The decrease in force of contraction was significant ($p < 0.05$) at concentrations >0.001 μ*M* for nifedipine and >0.01 μ*M* for verapamil. In contrast, concentrations >10 μ*M* were necessary to observe a significant negative inotropic effect of mibefradil.

The EC_{50} values for the negative inotropic effect were 0.1 (0.05–0.2) μ*M* for nifedipine, 0.6 (0.3–1.2) μ*M* for verapamil, and 18.5 (6.97–49) μ*M* for mibefradil (Fig. 2, Table 1).

The basal force of contraction was depressed by ~90% at concentrations >10 μ*M* of nifedipine and at concentrations >30 μ*M* of verapamil. In contrast, mibefradil, even at the maximum concentration of 100 μ*M*, depressed basal force of contraction only by ~10% (Fig. 1B).

As expected, addition of calcium to the organ bath led to a concentration-dependent increase in force of contraction. Only after pretreatment with the calcium antagonists nifedipine and verapamil was a significant rightward shift of the concentration–response curve for calcium observed compared with control. No significant rightward shift was observed after pretreatment with mibefradil. Moreover, only in the presence of nifedipine and verapamil was a significant depression of maximal inotropic effects of calcium observed (Fig. 3). The absolute value for the maximal increase in force of contraction induced by $CaCl_2$ (15 m*M*) was 6.9 (±1.4) mN after pretreatment with mibefradil, 3.5 (±0.7) mN after pretreatment with verapamil, and 1.7 (±0.3) mN after pretreatment with nifedipine, respectively. There were no significant differences in basal force of contraction, negative inotropic effects of the various calcium antagonists, or increase in force of contraction induced by $CaCl_2$ between ventricular tissue from moderately failing (NYHA Classes II–III) or terminally failing hearts (NYHA Class IV).

DISCUSSION

The therapeutic use of L-type calcium antagonists in the treatment of cardiovascular diseases is limited because of their negative effects on cardiac contractility be-

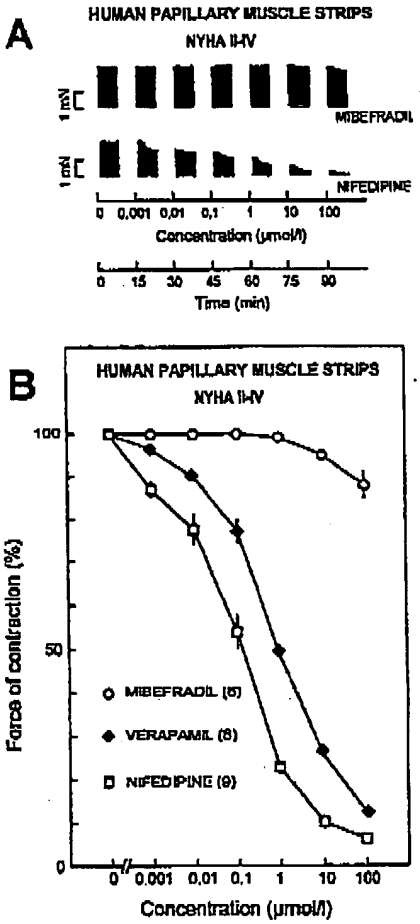


FIG. 1. Cumulative concentration–response curves for the effects of mibefradil, verapamil, and nifedipine on force of contraction in isolated, electrically driven human left ventricular papillary muscle strips. Original recordings (A) of isometric force of contraction after application of mibefradil (upper) and nifedipine (lower) and a summary of the results (B) are shown. Ordinate in B, force of contraction in percentage of predrug value. Abscissa in B, concentration of drug (in μM). Number of strips is shown in brackets.

cause of an impairment of transmembranous calcium transport in cardiomyocytes (14).

The T-type calcium channel, which is less prevalent and not completely understood, is found in relatively high density in spontaneously active vascular smooth-muscle cells but only rarely in myocardial tissue (15,16). Interestingly, Richard et al. (17) and Neveu et al. (18) showed that T-type calcium channels are not found in freshly dissociated rat aortic smooth-muscle cells but were expressed transiently between 5 and 15 days in cultured cells, during which time the cells were proliferating. These observations provide evidence that T-type calcium channel expression is important for cell proliferation.

The novel calcium antagonist mibefradil has been suggested to be more selective for vascular smooth-muscle

TABLE 1. *Negative inotropic potency of calcium antagonists in humans*

	In vitro EC_{50} (μM)	Mean TAPC (μM)	Therapeutic index	Relative potency
Mibefradil	18.5	0.87[a]	21.26	0.20
Nifedipine	0.1	0.14[b]	0.71	6.04
Verapamil	0.6	0.14[c]	4.29	1.0

Therapeutic index was calculated from the ratio of in vitro EC_{50} to mean TAPC. Relative potency of each drug was calculated from the therapeutic index of verapamil (1.0).

TAPC, therapeutic active plasma concentration; EC_{50}, 50% effective concentration.

[a]Clozel et al., 1991 (20).
[b]Henry, 1980 (14).
[c]McAllister and Kirsten, 1982 (19).

cells because of a preferential inhibition of T-type calcium channels (12). We might expect mibefradil to have fewer cardiodepressant side effects because of a higher selectivity for vascular smooth-muscle cells. In accordance with this theory, it has been shown in isolated vascular and cardiac muscle preparations, as well as in isolated perfused hearts from rats and guinea pigs, that mibefradil is more potent in increasing coronary artery flow (EC_{50} = 54 nM) than in suppressing myocardial contractility [50% inhibitory concentration (IC_{50}) = 14.000 nM] (11). Thus mibefradil shows an apparent preference for vascular tissue.

In this study, the inotropic effects of nifedipine, verapamil, and mibefradil were compared in vitro. All calcium antagonists exerted concentration-dependent negative inotropic effects in isolated, electrically driven human left ventricular papillary muscle strips (Fig. 1B). The EC_{50} values of the negative inotropic effects of the various calcium antagonists were found in the following

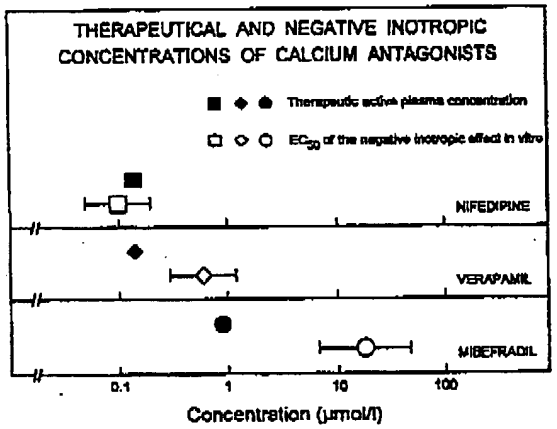


FIG. 2. Comparison of the therapeutic active plasma concentrations in humans after oral administration of nifedipine, verapamil, and mibefradil (solid symbols) with the effective concentrations for half-maximal effect (EC_{50}) of negative inotropic effects on force of contraction in isolated, electrically driven human left ventricular papillary muscle strips in vitro (open symbols).

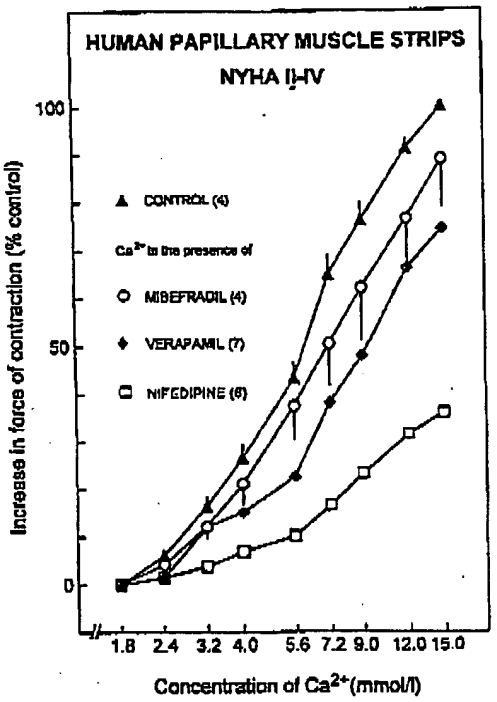


FIG. 3. Cumulative concentration–response curves for the effects of $CaCl_2$ in the presence of calcium antagonists, which depressed force of contraction in isolated, electrically driven human left ventricular papillary muscle strips by −90% with nifedipine (10 μM) and verapamil (30 μM) but only −10% in the presence of 100 μM mibefradil. Ordinate, increase in force of contraction measured as percentage of control value. Abscissa, concentration of $CaCl_2$ (in mM).

rank order of potency: nifedipine > verapamil >> mibefradil (Fig. 2, Table 1). Thus the negative inotropic potency of nifedipine and verapamil is much higher than of mibefradil. The concentration-dependent response to each of the various calcium antagonists was identical in specimens obtained from NYHA Classes II–IV patients. This may indicate that the detrimental negative inotropic effects can occur in even the moderately failing heart. The different rank order of potency becomes evident also if the effect of the calcium channel antagonists on the concentration-dependent increase in force of contraction in response to calcium is considered. Only pretreatment with nifedipine and verapamil led to a significant rightward shift of the inotropic concentration–response curves and a depression of the maximal effects of calcium. These observations strengthen the hypothesis that a T-type selective calcium channel antagonist acts on vascular smooth-muscle cells rather than on myocardial tissue.

To investigate whether the rank order of potency of the calcium antagonists examined in this study is relevant in vivo, we compared the EC_{50} values with the mean therapeutic active plasma concentrations (TAPCs) after oral application of these compounds (Fig. 2, Table 1).

Oral administration of therapeutic doses of 10–20 mg nifedipine led to a mean TAPC of 0.14 μM (14), and therapeutic doses of 120 mg verapamil, also to a mean TAPC of 0.14 μM (19) (Fig. 2, Table 1). In clinical trials in humans (20), the therapeutic oral dose of mibefradil was 120 mg in the treatment of arterial hypertension, leading to a mean TAPC of 0.87 μM (Fig. 2, Table 1). For nifedipine, the EC_{50} value occurred at a concentration (0.1 μM), which is less than the mean TAPC (0.14 μM). Thus nifedipine offers the least favorable balance between therapeutic and negative inotropic effects. In contrast, the EC_{50} value of the negative inotropic effect of mibefradil in vivo is 21.26 times higher than its mean TAPC (Table 1).

Expressing the difference (as a measure of the therapeutic index) between mean TAPC and EC_{50} values for cardiodepressant effects by calculating the ratio between both parameters, a rank order of the various calcium antagonists with respect to their advantage in the therapy of patients with heart failure can be established: mibefradil (21.26) >> verapamil (4.29) > nifedipine (0.71) (Table 1). This rank order of potency should be taken into consideration when patients with impaired myocardial function are treated with calcium antagonists.

The use of mibefradil, at least with respect to negative inotropic effects at relevant concentrations, could be advantageous compared with established calcium antagonists like nifedipine or verapamil. The novel calcium antagonist could be a key substance for the development of a new generation of calcium antagonists, which can be used in the therapy of a variety of cardiovascular diseases.

Acknowledgment: This work was supported by the Deutsche Forschungsgemeinschaft, Cardiovascular Research Foundation, and a research grant from Hoffmann-La Roche AG (Basel, Switzerland). Michael Böhm is a recipient of the Gerhard Hess program of the Deutsche Forschungsgemeinschaft. This work contains parts of the doctoral thesis of Bodo Cremers (University of Cologne, in preparation).

REFERENCES

1. Elkayam U, Amin J, Mehra A, Vasquez J, Weber L, Rahimtoola SH. A prospective, randomized, double-blind, crossover study to compare the efficacy and safety of chronic nifedipine therapy with that of isosorbide dinitrate and their combination in the treatment of chronic congestive heart failure. *Circulation* 1990;82:1954–61.
2. Furberg CD, Psaty BM, Meyer JV. Nifedipine: dose-related increase in mortality in patients with coronary heart disease. *Circulation* 1995;92:1326–31.
3. Waters D. Proischemic complications of dihydropyridine calcium channel blockers. *Circulation* 1991;84:2598–600.
4. Schwinger RHG, Böhm M, Erdmann E. Negative inotropic properties of isradipine, nifedipine, diltiazem, and verapamil in diseased human myocardial tissue. *J Cardiovasc Pharmacol* 1990;15:892–9.
5. Yasky J, Ferrario CM. Nocturnal hypotension and ACE inhibitors. *J Hum Hypertens* 1993;7:299–304.
6. Becker RC, Caputo R, Ball S, Corrao JM, Baker S, Gore JM. Hemorrhagic potential of combined diltiazem and recombinant tissue-type plasminogen activator administration. *Am Heart J* 1993;126:11–4.

7. Packer M. Pathophysiological mechanisms underlying the adverse effects of calcium channel-blocking drugs in patients with chronic heart failure. *Circulation* 1989;80:59–67.
8. Ferlinz J, Citron PD. Hemodynamic and myocardial performance characteristics after verapamil use in congestive heart failure. *Am J Cardiol* 1983;51:1339–45.
9. Yusuf S, Held P, Furberg C. Update of effects of calcium antagonists in myocardial infarction or angina in light of the second Danish Verapamil Infarction Trial (Davit-II) and other recent studies. *Am J Cardiol* 1991;67:1295–7.
10. Dibner-Dunlap ME, Thames MD. Abnormalities of baroflex control in heart failure. *Heart Failure* 1990;6:12–6.
11. Osterrieder W, Holck M. In vitro pharmacologic profile of Ro 40-5967, a novel Ca^{2+} channel blocker with potent vasodilator but weak inotropic action. *J Cardiovasc Pharmacol* 1989;13:754–9.
12. Mishra SK, Hermsmeyer K. Selective inhibition of T-type Ca^{2+} channels by Ro 40-5967. *Circ Res* 1994;75:144–8.
13. Böhm M, Diet F, Pieske B, Erdmann E. Screening of positive inotropic agents in isolated cardiac preparations from different sources. *J Pharmacol Methods* 1989;21:33–4.
14. Henry PD. Comparative pharmacology of calcium antagonists: nifedipine, verapamil and diltiazem. *Am J Cardiol* 1980;46:1047–58.
15. Bean BP. Classes of calcium channels in vertebrate cells. *Annu Rev Physiol* 1989;51:367–84.
16. Hermsmeyer K. Differences of calcium channels in vascular muscle in hypertension. *Am J Hypertens* 1991;4:412–5S.
17. Richard S, Neveu D, Carnac G, Bodin P, Travo P, Nargeot J. Differential expression of voltage-gated Ca^{2+}-currents in cultivated aortic myocytes. *Biochim Biophys Acta* 1992;1160:95–104.
18. Neveu D, Quignard JF, Fernandez A, Richard S, Nargeot J. Differential β-adrenergic regulation and phenotypic modulation of voltage-gated calcium currents in rat aortic myocytes. *J Physiol* 1994;479(2):171–82.
19. McAllister RG, Kirsten EB. The pharmacology of verapamil. IV. Pharmakokinetics and drug effects after single intravenous and oral doses in normal subjects. *Clin Pharmacol Ther* 1982;31:418–26.
20. Clozel JP, Osterrieder W, Kleinbloesem CH, et al. Ro 40-5967: a new nondihydropyridine calcium antagonist. *Cardiovasc Drug Rev* 1991;9:4–17.

.05). Maximal
S diet group
in the capsules
$^{-4}$M), a specific
dosterone when
d with 10^{-4}M 5-
erone secretion.
' present in the
of the rat adrenal
erulosa/capsular
id its ability to
n and attenuated
L-AAAD could
dium deficiency
ent in blood at
renal cortex.

1989 J Endocr

15-649.

3-1453.

2 Neuroscience

ochem Biophys

J. 1993 Brain

:8 J Biochem

47-1167.

ENDOCRINE RESEARCH, 22(4), 579-588 (1996)

SOURCES AND SITES OF ACTION OF CALCIUM IN THE REGULATION OF ALDOSTERONE BIOSYNTHESIS

M.F. Rossier, M.M. Burnay, Y. Brandenburger, N. Cherradi, M.B. Vallotton and A.M. Capponi

Division of Endocrinology and Diabetology, Department of Internal Medicine, University Hospital, Geneva, Switzerland

ABSTRACT

The role of free calcium as a crucial intracellular messenger in the stimulation of aldosterone biosynthesis by various agonists is well established. Using electropermeabilized or Ca^{2+}-clamped adrenal zona glomerulosa (ZG) cells, we have previously shown that Ca^{2+} entry into the mitochondrial matrix is required for the activation of steroidogenesis. We now describe the use of various strategies to answer the following questions: 1. Which pathway does Ca^{2+} follow before triggering steroidogenesis? 2. Which step of steroidogenesis is under the control of Ca^{2+}? The first approach combined the patch-clamp method, in the perforated patch configuration, with microfluorimetry of Ca^{2+}; in the second approach, ZG cells were transiently transfected with a chimeric cDNA encoding for the calcium-sensitive photoprotein aequorin linked to a mitochondrial targeting presequence; in a third approach, ZG mitochondria were isolated and fractionated into outer membranes, contact sites and inner membranes and the effect of prior exposure of the ZG cells to a physiologically elevated intracellular calcium concentration or to angiotensin II (Ang II) on cholesterol content was then examined in those three mitochondrial fractions. The results of these combined approaches allow us to propose the following scheme: The source of calcium which is predominantly responsible for mediating the steroidogenic effect of potassium appears to be funneled through the T-type calcium channels to close proximity of the mitochondria. This signal, as well as that triggered by Ang II, appears to be relayed within the mitochondrial matrix. This rise of mitochondrial calcium is associated with a transfer of free cholesterol from the outer to the inner mitochondrial membrane, via the contact sites. Thus the main role of the calcium messenger is to promote intramitochondrial cholesterol transfer and supply to the $P450_{scc}$ enzyme.

INTRODUCTION

Angiotensin II (Ang II) and potassium (K^+) are, along with ACTH, the most important stimulators of aldosterone biosynthesis in the adrenal zona glomerulosa cell. The cytosolic free calcium ($[Ca^{2+}]_c$) plays, as a messenger triggered by Ang II or K^+, a central role in the stimulation of aldosterone synthesis in those cells (1-3). In fact, the rise of $[Ca^{2+}]_c$ has been found previously, in permeabilized or in Ca^{2+}-clamped ZG cells, to be necessary and sufficient for the acute activation of aldosterone synthesis (4,5). Ang II and K^+ are known to activate the Ca^{2+} messenger system through different mechanisms, both relying upon the presence of extracellular Ca^{2+}. The Ang II-induced $[Ca^{2+}]_c$ rise is biphasic, with a transient release of Ca^{2+} from intracellular stores followed by a sustained influx across the cell membrane. The latter process results from the combined effects of cell membrane depolarization, with subsequent activation of the calcium influx pathway through voltage dependent T-type and L-type calcium channels, and of activation of the capacitative calcium influx pathway (6,7). The effect of the rise of ambient Ca^{2+} upon aldosterone synthesis in permeabilized ZG cells has been found to be abolished by ruthenium red, a blocker of the mitochondrial calcium uniport (4,8), a first evidence that calcium must enter mitochondria to activate steroidogenesis. Yet, at that time, the sites of action of calcium and its specific molecular targets in the steroidogenic cascade leading to aldosterone production were unknown. Recently, our group has been able to limit the target domain for Ca^{2+} to the earliest mitochondrial steps of steroidogenesis (5). The rate-limiting step of steroidogenesis is the delivery of cholesterol from the outer to the inner mitochondrial membrane, which occurs after the mobilization of cholesterol from lipid droplets and precedes the first enzymatic step. This transport is also known to require *de novo* protein synthesis. Among the hormone-induced, cycloheximide-sensitive mitochondrial proteins, the Steroidogenic Acute Regulatory (StAR) protein, recently cloned, has been proposed as an essential mediator of the acute hormone-induced regulation of steroid synthesis (9). The purpose of our most recent work was to define more precisely the main sources of the Ca^{2+} responsible for aldosterone biosynthesis and to pinpoint the site of action of Ca^{2+} in the ZG cells.

ACTH, the most
a glomerulosa cell.
by Ang II or K^+, a
s (1-3). In fact, the
Ca^{2+}-clamped ZG
losterone synthesis
m through different
he Ang II-induced
intracellular stores
rocess results from
nt activation of the
calcium channels,
ie effect of the rise
s has been found to
um uniport (4,8), a
idogenesis. Yet, at
ular targets in the
own. Recently, our
liest mitochondrial
s is the delivery of
ch occurs after the
st enzymatic step.
nong the hormone-
eroidogenic Acute
essential mediator
he purpose of our
e Ca^{2+} responsible
in the ZG cells.

MATERIALS AND METHODS

Preparation of Purified Zona Glomerulosa Cells. Bovine adrenal glands were obtained from a local slaughterhouse and glomerulosa cells were prepared by enzymatic dispersion with dispase, purified on Percoll density gradients, and used either freshly prepared or after three days in primary culture (10).

Patch Clamp Measurements and Measurement of Cytosolic Calcium. The activity of voltage-activated calcium channels was recorded under voltage clamp in the perforated patch or whole cell configurations. For the microfluorimetric measurement of cytosolic calcium concentration the fluo-3 or fura-2 fluorescent dyes were used (7).

Plasmid Construct and Transfections. The plasmid used was kindly donated by Drs R. Rizzuto and T. Pozzan, University of Padova, Italy. The construct, described elsewhere (11), consists of the cDNA encoding the first 33 amino acids of human cytochrome c oxidase subunit VIII fused in frame with a cDNA coding for all but the first (start codon) amino acid of the mature chemiluminescent aequorin polypeptide. This chimeric cDNA was inserted into the expression vector pcDNAI (InVitrogen). Purified bovine zona glomerulosa cells in primary culture were transiently transfected using either the lipopolyamine Transfectam® (Promega, USA) or calcium phosphate. Luminescence of aequorin was measured as described elsewhere (12).

Preparation and Identification of Submitochondrial Fractions. After a 2h incubation period with either a calcium-clamp or Ang II, the glomerulosa cells were homogenized and their mitochondria obtained by differential centrifugation. The mitochondria were then disrupted by swelling and sonication and the membranous particles submitted to fractionation by ultracentrifugation on a linear sucrose density gradient. The cholesterol content of each submitochondrial fraction of the gradient was determined by a coupled cholesterol oxidase-peroxidase assay (13).

Steroid Measurement. The steroid content of the incubation media was measured by radioimmunoassay, using either a commercially available kit (aldosterone, DSL, Webster, TX), or[^{3}H]pregnenolone and an antibody kindly provided by Professor P. Vecsei (University of Heidelberg, Germany) (14).

RESULTS

Sources of Calcium Responsible for Steroidogenesis. We first examined the relative contributions of T- and L-type calcium channels to the elevations of cytosolic calcium and aldosterone synthesis induced by potassium. In preliminary experiments we observed that the rise in $[Ca^{2+}]_c$ elicited upon single cell depolarization (in the perforated patch configuration of the patch clamp technique) was completely abolished after selective inhibition of the L-type Ca^{2+} currents. This finding suggested that L-type channels are exclusively responsible for the Ca^{2+} signal detected in the cytosol of glomerulosa cells stimulated with extracellular potassium. By applying a progressive and controlled ramp depolarization to single zona glomerulosa cells, it is possible to activate separately first the T-type calcium channels and then the L-type channels, which can be selectively inhibited or stimulated with pharmacological agents. Both current amplitudes can thus be represented as a function of the concentration of the various agents. Nifedipine, at 100 nM, was found to inhibit selectively the L-type channels (33% of the control compared to 93% of the control for the T-type calcium channels). However, at concentrations above 1 mM, nifedipine also markedly affected the T-type calcium channels. The dihydropyridine agonist, BayK 8644, at 100 nM, activated selectively L-type calcium channels (220% of the control compared to 115% of the control for the T-type calcium channels). The compound zonisamide at 1 mM had only a slight preferential effect on the T-type calcium channels (30% of the control compared to 55% of the control for L-type calcium channels). Tetrandrine, at 10 μM, affected essentially the T-type calcium channels (52% of the control) but not the L-type calcium channels (more than 90% of the control). Nicardipine, at 10 nM, had a slight stimulatory action on T-type calcium channels (130% of the control) but inhibited L-type channels (59% of the control). Using these agents, the effect of potassium (12 mM) on the $[Ca^{2+}]_c$ measured in cell populations with the fura-2 fluorescent dye and on aldosterone production could be dissociated. Nifedipine, which was found to selectively inhibit L-type calcium channels, induced a marked decrease of $[Ca^{2+}]_c$ (21% of the control) but minimally affected aldosterone production (70% of the control). BayK 8644, which stimulated the L-type calcium channels, increased $[Ca^{2+}]_c$ (225% of the control) but, simultaneously,

ed the relative
tosolic calcium
xperiments we
ization (in the
ietely abolished
sted that L-type
the cytosol of
progressive and
sible to activate
is, which can be
rrent amplitudes
various agents.
ıels (33% of the
ls). However, at
T-type calcium
ɔd selectively L-
ontrol for the T-
d only a slight
ompared to 55%
fected essentially
calcium channels
ulatory action on
ınels (59% of the
$Ca^{2+}]_c$ measured
production could
: L-type calcium
ol) but minimally
ich stimulated the
t, simultaneously,

inhibited aldosterone production (70% of the control). Zonisamide and tetrandrine, through selective inhibition of the T-type calcium channels, lowered aldosterone production (87% and 59% of the control, respectively) with no effect on $[Ca^{2+}]_c$ for zonisamide (102% of the control). Nicardipine, which selectively inhibited L-type calcium channels, lowered $[Ca^{2+}]_c$ (27% of the control) but not aldosterone production (125% of the control).

Site of Action of Calcium in the Activation of Steroidogenesis. We next examined how changes in $[Ca^{2+}]_c$ induced by either Ang II or K^+ were relayed into the mitochondrial matrix where calcium is expected to exert its effect on the first rate-limiting step of steroidogenesis. In glomerulosa cells transfected with the chimeric aequorin targeted to the mitochondria, we could observe upon stimulation with Ang II (10 nM) a prominent transient peak of mitochondrial calcium reaching close to 5000 nM, well above the values recorded in the cytosol of cells in which the aequorin was confined to the cytosol, followed by a prolonged plateau significantly higher than the basal value. In the absence of extracellular calcium, Ang II still induced the first transient peak of mitochondrial free calcium concentration which, however, was no longer followed by a plateau. When calcium was reintroduced into the medium bathing the cells, only a small sustained increase of mitochondrial free calcium concentration occurred. To prove that the luminescent probe aequorin had indeed been targeted and was confined to the mitochondria, the transfected cells were superfused with the uncoupling agent, FCCP. Under this circumstance, a complete suppression of the Ang II-induced mitochondrial calcium response was observed whereas the cytosolic calcium response to Ang II was not affected. Rising the extracellular potassium concentration from 3 to 13 mM induced a rapid elevation of mitochondrial free calcium concentration, which remained at the level of approximately 450 nM as long as high K^+ was present and returned to basal levels upon lowering the K^+ concentration to 3 mM or exposing the cells to nifedipine (200 nM).

We addressed in parallel the question of whether calcium or Ang II affected the distribution of cholesterol between the outer and the inner mitochondrial membranes. For that purpose the bovine adrenal glomerulosa cells were first exposed either to Ang II (10 nM) or to a high intracellular calcium-clamp in the presence of 2 μM ionomycin,

resulting in a $[Ca^{2+}]_c$ of 600 nM. Aminoglufhetimide (500 mM) was included in the medium to prevent cholesterol side chain cleavage by the $P450_{scc}$. After a 2h incubation period at 37°C under those conditions, the cells were sedimented and the mitochondria fractionated as indicated under Materials and Methods. Three mitochondrial membrane populations could be distinguished on the basis of marker enzyme activities: a first peak of monoamine oxidase activity, representing the outer membranes, a second membrane population with the highest concentration of cytochrome c oxidase activity, corresponding to the inner membranes, and a membrane population of intermediate density possessing both of these enzymatic activities and, in addition, a peak of nucleoside diphosphate kinase activity, which is characteristic of the mitochondrial intermembrane contact sites. Cholesterol content was determined in those membrane populations. In the mitochondrial fractions obtained from cells exposed to a high calcium clamp, one could observe a decrease of cholesterol content in the outer membranes and a rise of cholesterol content in the contact sites, extending to the inner membranes. In the mitochondrial fractions obtained from cells exposed to Ang II, again a net increase in the cholesterol content of the contact sites and the inner membranes was observed. In contrast to the response to calcium, no decrease of cholesterol content occurred in the outer membranes. Inhibition of *de novo* protein synthesis by cycloheximide prevented the responses to both calcium and Ang II.

DISCUSSION

Bovine glomerulosa cells are characterized by the presence of both low threshold T-type and high threshold L-type calcium channels (10,15). Potassium ion depolarizes the cell membrane and thereby opens voltage-operated calcium channels. Glomerulosa cells display an exquisite sensitivity to extracellular K^+, suggesting that low threshold T-type calcium channels are primarily involved when K^+ concentration is increased within the physiological range (16). By contrast, Ang II is known to release calcium from intracellular Ca^{2+} pools, through generation of inositol 1,4,5-trisphosphate (17). In addition, Ang II has been shown to induce depolarization by inhibiting some potassium conductances and thus could also stimulate Ca^{2+} influx through T-type calcium channels (3). Another pathway for calcium influx resulting from intracellular Ca^{2+} pool emptying

as included in the
ter a 2h incubation
d the mitochondria
ondrial membrane
ivities: a first peak
second membrane
oxidase activity,
on of intermediate
ldition, a peak of
the mitochondrial
n those membrane
d to a high calcium
r membranes and a
membranes. In the
net increase in the
was observed. In
ent occurred in the
ieximide prevented

th low threshold T-
ion depolarizes the
. Glomerulosa cells
ow threshold T-type
ncreased within the
easc calcium from
phosphate (17). In
ing some potassium
pe calcium channels
Ca^{2+} pool emptying

has been described in glomerulosa cells (6). This capacitative Ca^{2+} influx, originally observed in nonexcitable cell types (18), can be activated by low concentrations of thapsigargin, an agent which leads to emptying of Ca^{2+} from intracellular stores through inhibition of the associated Ca^{2+}/Mg^{2+}-ATPase. Nicardipine, at concentrations which inhibit both T- and L-type voltage-operated calcium channels activity, as measured by the patch-clamp technique, inhibited completely the rise of $[Ca^{2+}]_c$ induced by K^+. By contrast, nicardipine did not affect the response to thapsigargin and reduced by only 22% the calcium influx stimulated by Ang II. This nicardipine-insensitive component of the latter response was abolished after emptying the intracellular Ca^{2+} stores with thapsigargin. As a proof of the secondary role of voltage-operated calcium channels in the response to Ang II, nicardipine only partially inhibited (40%) the Ang II-induced aldosterone production, at a concentration which completely prevented the steroidogenic response to K^+ (6). The data summarized in the present paper suggest a direct link between T-type calcium channel activity and aldosterone biosynthesis in response to K^+. We had already previously shown that tetrandrine inhibits with a similar potency T-type calcium channel activity and aldosterone production (10). Furthermore, using various inhibitors of voltage-operated calcium channels, at concentrations affecting selectively the activity of T- or L-type calcium channels, we could demonstrate a dissociation between $[Ca^{2+}]_c$ changes and aldosterone production. Taken together, these results suggest a scheme according to which Ca^{2+} entering the cell through T-type channels is the principal mediator of intramitochondrial aldosterone biosynthesis (Fig. 1).

We have previously shown, using electropermeabilized glomerulosa cells, that rising ambient Ca^{2+} was a necessary and sufficient condition for aldosterone production. Furthermore, we observed that ruthenium red, an inhibitor of mitochondrial calcium uptake, abolished the stimulation induced by a rise of ambient Ca^{2+}, an indication that Ca^{2+} must enter the mitochondria to exert its effect on steroidogenesis (4). More recently, we performed studies in Ca^{2+}-clamped bovine adrenal zona glomerulosa cells, measuring in parallel steroid synthesis and $[Ca^{2+}]_c$, and reported that Ca^{2+} levels regulated the production of both pregnenolone and aldosterone, but not the conversion of 11-deoxycorticosterone into aldosterone (5). In addition, Ca^{2+} changes did not affect the formation of pregnenolone from freely diffusible hydroxycholesterol. The latter result indicates that Ca^{2+} acts at a step upstream of cholesterol side-chain cleavage. *De novo*

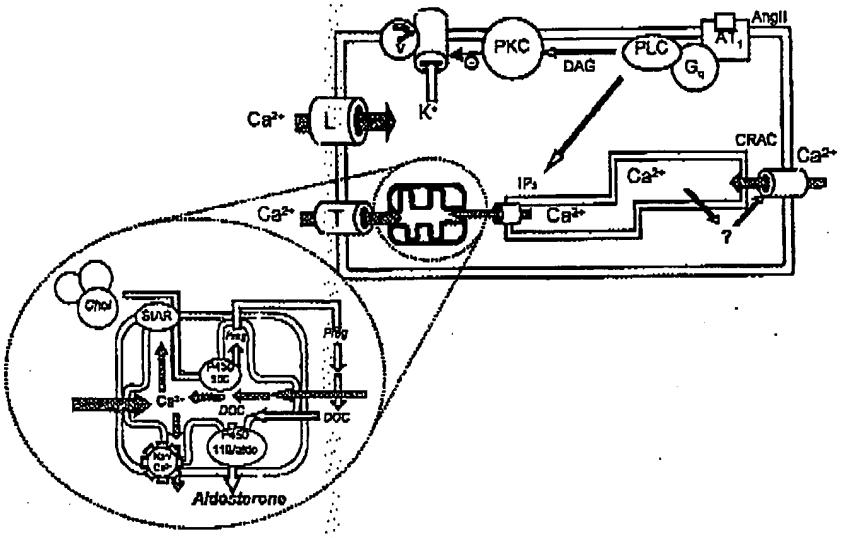
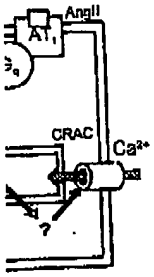


FIGURE 1

Calcium-induced steroidogenesis in adrenal glomerulosa cells. The abbreviations used are the following: Ang II, angiotensin II; AT_1, angiotensin II receptor subtype 1; G_q, GTP binding protein; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; V, membrane potential; L and T, L-type and T-type voltage-operated Ca^{2+} channels; IP_3, inositol 1,4,5-trisphosphate; CRAC, Ca^{2+} release-activated Ca^{2+} channel; Chol, cholesterol; StAR, steroidogenic acute regulatory protein; $P450_{scc}$ and $P450_{11\beta/aldo}$, cytochrome P450 side chain cleavage and aldosterone synthase enzyme; Preg, pregnenolone; DOC, deoxycorticosterone; Na^+/Ca^{2+}, mitochondrial sodium/calcium exchanger.

protein synthesis appeared to be involved, as cycloheximide, an inhibitor of protein translation, blocked the Ca^{2+}-induced pregnenolone production (5).

Glomerulosa cells transfected with mitochondrial aequorin allowed us to demonstrate that Ang II elicited a transient rise of mitochondrial $[Ca^{2+}]$ well above that observed in the cytosolic compartment. This could suggest the existence of a restricted microdomain of high $[Ca^{2+}]_c$ in close proximity of the mitochondria, due to the presence of Ca^{2+} release sites [Ins(1,4,5)P_3 receptors]. Moreover, the close correlation between T channel activity and aldosterone secretion could be explained by the fact that Ca^{2+} entering the cell through these channels is funneled to close proximity of the



mitochondria and that this signal is specifically relayed into the mitochondrial matrix (Fig. 1).

The question of the exact site of action and specific molecular targets of calcium was then examined using fractionation of mitochondrial membranes. As mentioned above, previous studies from our group in electropermeabilized or in Ca^{2+}-clamped cells pointed to the very early mitochondrial steps of steroidogenesis as likely targets for calcium. We examined whether calcium or Ang II affected the distribution of cholesterol between the outer and the inner membranes. Indeed, a rise of $[Ca^{2+}]_c$ or exposure to Ang II led to a transfer of free cholesterol from the outer membranes to the contact sites and the inner mitochondrial membranes. These effects were prevented by exposure of the cells to cycloheximide. This indicates that *de novo* protein synthesis was required for this process. One of the most likely candidate protein is the recently cloned StAR protein (9).

In conclusion, Ca^{2+} enters the zona glomerulosa cells through distinct pathways, depending upon the stimulus applied, and the local rise of $[Ca^{2+}]_c$ is relayed into the mitochondrial matrix. One of the major functions of Ca^{2+} appears to be the facilitation of cholesterol transfer, via contact sites, between the outer and the inner mitochondrial membranes, thus increasing cholesterol supply to the $P450_{scc}$ enzyme.

bbreviations used
or subtype 1; G_q,
C, protein kinase
d Ca^{2+} channels;
$^{2+}$ channel; Chol,
nd $P450_{11\beta/aldo}$
e enzyme; Preg,
l sodium/calcium

hibitor of protein

l allowed us to
$^{2+}]$ well above that
nce of a restricted
ondria, due to the
e close correlation
ned by the fact that
e proximity of the

ACKNOWLEDGEMENTS

This work was supported by Swiss National Science Foundation grants No. 32-39277.93 and 31-42178.94. MFR is a recipient of a grant from the Prof. Max Cloetta Foundation.

REFERENCES

1. Barrett PQ, Bollag WB, Isales CM, McCarthy RT, Rasmussen H. 1989 Endocr Rev 10:496-518.
2. Spät A, Enyedi P, Hajnoczky G, Hunyady L. 1991 Exp Physiol 76:859-885.
3. Capponi AM, Python CP, Rossier MF. 1994 Endocrine 2:579-586.
4. Capponi AM, Rossier MF, Davies E, Vallotton MB. 1988 J Biol Chem 263:16113-16117.
5. Python CP, Laban OP, Rossier MF, Vallotton MB, Capponi AM. 1995 Biochem J 305:569-576.
6. Burnay MM, Python CP, Vallotton MB, Capponi AM, Rossier MF. 1994 Endocrinology 135:751-758.

7. Rossier MF, Burnay MM, Vallotton MB, Capponi AM. 1996 Endocrinology (in press)
8. Rossier MF, Krause KH, Lew PD, Capponi AM, Vallotton MB. 1987 J Biol Chem 262:4053-4058.
9. Stocco DM, Clark BJ. 1996 Endo Rev 17:221-244.
10. Rossier MF, Python CP, Capponi AM, Schlegel W, Kwan CY, Vallotton MB. 1993 Endocrinology 132:1035-1043.
11. Rizzuto R, Simpson AWM, Brini M, Pozzan T. 1992 Nature 358:325-327.
12. Rutter GA, Theler JM, Murgia M, Wollheim CB, Pozzan T, Rizzuto R. 1993 J Biol Chem 268:22385-22390.
13. Gamble W, Vaughan M, Kruth HS, Avigan J. 1978 J Lipid Res 19:1068-1070.
14. Abraham GE, Buster JE, Kyle FW, Corrales PC, Teller RC. 1973 J Clin Endocrinol Metab 37:40-45.
15. Matsunaga H, Maruyama Y, Kojima I, Hoshi T. 1987 Pflügers Arch 408:351-355.
16. Barrett PQ, Isales CM, Bollag WB, McCarthy RT. 1991 Am J Physiol 261:F706-F719.
17. Rossier MF, Capponi AM, Vallotton MB. 1989 J Biol Chem 264:14078-14084.
18. Putney JW. 1990 Cell Calcium 11:611-624.

Seizure 1996; 5: 115–119

Mechanisms of T-type calcium channel blockade by zonisamide

MASAO KITO,* MITSUO MAEHARA† & KAZUYOSHI WATANABE‡

*Department of Pediatrics, Minami Seikyo Hospital, Nagoya, †Department of Pediatrics, Chubu National Hospital, Obu and ‡Department of Pediatrics, Nagoya University School of Medicine, Nagoya, Japan

Correspondence to: M. Kito, Department of Pediatrics, Minami Seikyo Hospital, Miyoshi-cho 6-8, Minami-ku, Nagoya City, 457 Japan.

We investigated the effects of zonisamide, a new antiepileptic drug, on voltage-dependent T-type calcium current (I_{Ca}) in cultured neuroblastoma cells of human origin (NB-I). Zonisamide reduced T-type I_{Ca} in a concentration-dependent manner without evoking any change in its inactivation kinetics or voltage dependence of action. The mean percent reduction was 38.3 ± 5.8% at 50 μM. Further, zonisamide shifted the inactivation curve approximately 20 mV negative compared to the control. These resting blocking actions suggest that zonisamide shifts the channel population toward the inactivation state, allowing fewer channels to open during membrane depolarization. The blockade of T-type calcium channels by zonisamide could suppress an important component of inward current that underlies epileptiform cellular bursting, thereby inhibiting the spread of seizure activity.

Key words: zonisamide; absence seizure; calcium channel currents; whole-cell clamp recording; human neuroblastoma cell line.

INTRODUCTION

Zonisamide (ZNS; 1,2-benzisoxazole-3-methanesulfonamide; AD-810; CI-912), a derivative of 3-(sulfamoylmethyl)-1,2-benzisoxazole, is a member of novel class of anticonvulsants. ZNS has been proven effective in the treatment of both partial and generalized seizures[1]. Studies of its cellular mechanisms of action, especially its effects on ionic channels, have shown that ZNS blocks the sustained firing of action potentials recorded intracellularly in cultured spinal cord neurons[2], and that it affects the steady-state inactivation of sodium channels[3]. Recently, Suzuki *et al*[4] reported that ZNS blocked T-type calcium channels in cultured rat cerebral cortical neurons. We have also reported that ZNS reduces T-type I_{Ca} in human neuroblastoma cells[5]. Unfortunately, the mechanisms whereby ZNS exerts its action on calcium channels are incompletely understood. In this study, we examined the effects of ZNS on T-type I_{Ca} in the human neuroblastoma cell line NB-I. We used a whole-cell voltage-clamp technique, recording in regard to two points: (1) concentration dependent blockade by ZNS of T-type I_{Ca}, and (2) the effects of ZNS on T-type calcium channel inactivation.

METHODS

Cell culture

The human neuroblastoma cell line NB-I established by Miyake *et al* was used[6]. Cells were cultured in RPMI 1640 medium, pH 7.4, supplemented with 10% fetal calf serum at a temperature of 37°C. NB-I cells were replaced on a small glass-covered culture dish and incubated for two to seven days before use.

Recording conditions

Whole cell recording by the patch-clamp technique, was used to record T-type I_{Ca} in the neuroblastoma cell membrane under the voltage-clamp conditions[7]. The cut-off frequency of the recording system was 700 Hz. The recording chamber (bath volume 0.2 ml) in which NB-I cells were fixed was perfused via a gravity-fed

1059–1311/96/020115+05 $12.00/0

perfusion system at a rate of 2 ml/min. T-type I_{Ca} was evoked by applying step-depolarization of 400 ms duration from −100 mV to +80 mV in 10-mV steps from a holding potential of −80 mV. Experiments were carried out at room temperature (22–25°C).

The normal external medium used contained, in mM:NaCl 36.7, $BaCl_2$ 51.2, $MgCl_2$ 1.18, glucose 11.8, HEPES-Na 10.0, tetraethylammonium chloride (TEA) 23.6, and tetrodotoxin (TTX) 3×10^{-3}, at pH 7.4. The patch pipette was filled with a solution containing, in mM:Cs-ASP 106.2, CsCl 23.6, $MgCl_2$ 4.95, ATP-Na_2 4.95, EGTA 9.9, HEPES-Na 4.95, and $CaCl_2$ 1.26, at pH 7.0. The resistance of the patch pipette in the normal external solution was between 3 and 5 MΩ. Ba^{2+} was used in the normal external solution since it is more permeable to Ca^{2+} channels and the amplitude is easier to analyse than with Ca^{2+}[8].

ZNS was dissolved in dimethyl sulfoxide (DMSO) and this solution was added at various ZNS concentrations, to the normal external solution. The final concentration of DMSO was 0.5%, at which concentration DMSO inhibited T-type I_{Ca} by 4.3 ± 4.3% ($n = 5$). To differentiate the effects of DMSO and ZNS, the concentration of DMSO was maintained at 0.5% in all test solutions. The effect of 0.5% DMSO was taken into account when we calculated the percent inhibition in the experiment.

Approximately 50% of ZNS is bound to plasma proteins. Free plasma levels of ZNS are equal to those in cerebrospinal fluid (CSF). The physiologically relevant concentrations are presumed to be closer to the free brain concentration, which, in turn, is thought to be equivalent to CSF levels[9]. The therapeutic level of ZNS is 50 μM (21.2 μg/ml)[1]. In this study, we used ZNS at a concentration of 50 μM.

Data recording and analysis

The membrane currents recorded in the whole cell clamp mode, with a pre-amplifier (CEZ 2300; Nihon Kohden, Tokyo, Japan) were fed to a 12-bit analog-digital converter in a personal computer (IBM, USA). The computer was also used to control the membrane potential. Data were plotted as the current-voltage relationship (I–V curve) and the amplitude was analysed before and 5 minutes after the application of ZNS, and after ZNS had been washed out. Data were analysed with the PCLAMP version 5.51 (Axon Instruments, Inc., USA), and numerical values were expressed as means ± SEM. Wilcoxon analysis was used in the statistical analysis.

RESULTS

Characterization of T-type I_{Ca}

T-type I_{Ca} was activated by depolarizing potentials exceeding −50 mV and reached a maximum at 0 mV. T-type I_{Ca} was rapidly inactivated during depolarizing test potential and its time constant at −10 mV test potential was 22.5 ± 5.7 ms (Fig. 1). Ni^{2+} at 100 μM inhibited T-type I_{Ca} by 82.6 ± 15.3% ($n = 6$). On the other hand, Cd^{2+} had little effect on T-type I_{Ca} (11.4 ± 3.2% at 100 μM, $n = 7$). La^{3+} at 10 μM inhibited T-type I_{Ca} by 24.3 ± 5.5% ($n = 4$). Nifedipine[10] at 10 μM inhibited T-type I_{Ca} by 20.6 ± 0.6% ($n = 3$). ω-CgTX[11] 5 μM inhibited T-type I_{Ca} by 12.9 ± 7.6% ($n = 5$). At a concentration of 10 μM, Bay K 8644[12] did not enhance the T-type I_{Ca} ($n = 5$). T-type I_{Ca} was detected in 72.8% of NB-I cells examined ($n = 87$).

Effect of ZNS on T-type I_{Ca}

At a concentration of 100 μM, ZNS inhibited T-type I_{Ca} by 83.8%, but its inactivation kinetics was unchanged and the voltage dependence of current activation was not altered (Fig. 1). Figure 2 shows the results of the concentration-response experiments. ZNS reduction of T-type I_{Ca} was concentration-dependent. The percent inhibition exerted by 50 μM ZNS was 38.3 ± 5.8% ($n = 5$).

Voltage dependence of T-type calcium channel inactivation

Voltage dependence T-type calcium channel inactivation was tested with another series of experiments. I_{Ca} was evoked by applying 350-ms prepulse from a holding potential of −100 mV, between −120 mV and +40 mV, in 10-mV steps, followed by a test potential to 0 mV. Relative peak amplitudes of the inward currents were plotted against the voltage level during the prepulse. T-type calcium channel inactivation was strongly inhibited between −90 mV and −20 mV, and was completely inactivated at +10 mV prepulse. The data points for T-type I_{Ca} were fitted with a continuous smooth curve derived from the Boltzmann equation with a mid-point of

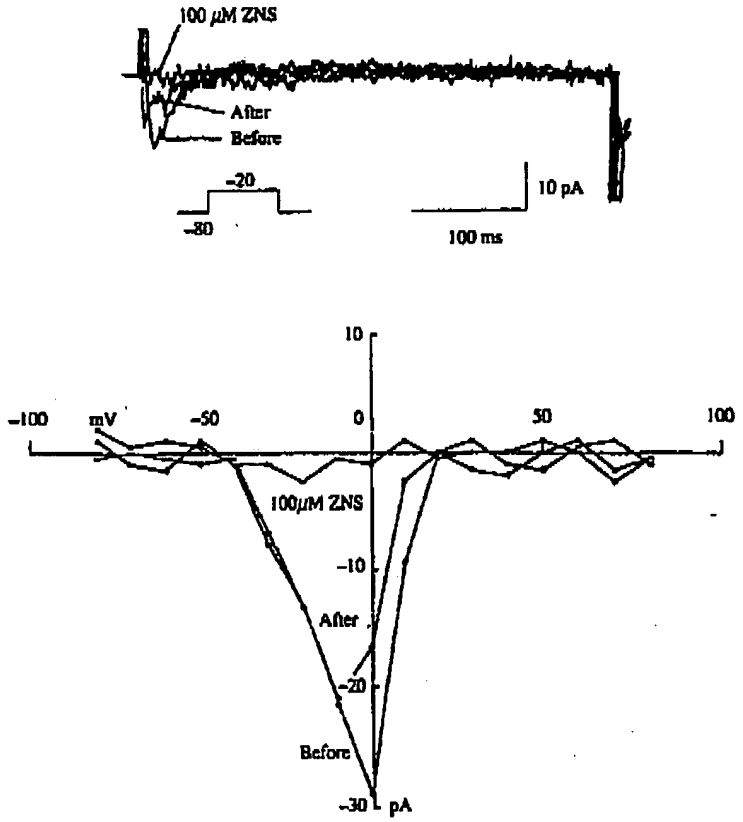


Fig. 1: Effects of ZNS on T-type I_{Ca} and on the current-voltage relationships of I_{Ca}. Typical inward currents evoked by applying step-depolarization from holding potentials of −80 mV to the test potentials indicated. The current was measured before and 5 minutes after the application of ZNS and 5 minutes after the wash out of ZNS. ZNS (100 μM) inhibited T-type I_{Ca} by 63.6%, but inactivation kinetics was unchanged and the voltage-dependence of current activation was not altered.

−50.4 ± 1.5 mV and slope parameter of 14.8 ± 1.1 mV ($n = 4$; Fig. 3). T-type calcium channel inactivation excrted by ZNS was strongly inhibited between −100 and −40 mV; and completely inactivated at 0 mV prepulse. The data points for T-type calcium channel inactivation by ZNS were also fitted with a continuous smooth curve derived from the Boltzmann equation, with a mid-point of −69.8 ± 2.1 mV and a slope parameter of 12.5 ± 0.8 mV ($n = 4$; Fig. 3). The membrane potential at half inactivation for ZNS was about 20 mV more negative than that for control ($P < 0.01$, Wilcoxon analysis).

DISCUSSION

ZNS, one of a novel class of of benzisoxazole anticonvulsants, has been proven effective in the treatment of simple/complex partial seizures and secondarily generalized tonic–clonic seizures, generalized tonic–clonic seizures, tonic seizures, atypical absence seizures and a combination of these[1]. The mechanism of action of ZNS, in particular, its effect on ionic channels, is incompletely understood. Schauf *et al*[3] reported that zonisamide enhanced slow medium inactivation in Myxicola. Suzuki *et al*[4] reported that zonisamide blocked T-type calcium channel in cultured neurons of rat cerebral cortex. Recently, we also reported that ZNS blocked T- and L-type I_{Ca} in cultured human neuroblastoma cells[5].

Recent reports have demonstrated that there are at least three types of Ca^{2+} channels in neuronal cells[8,10,13]. Calcium channels play important roles in the regulation of many cellular functions, including excitability, transmitter release, contraction, metabolism, and gene expressions[14]. The T-type calcium channels may be involved in a near-threshold membrane potential. For example, they may speed up the rate of depolarization to the threshold potential after neuronal hyperpolarization. Suzuki *et al*[15] reported that T-type Ca^{2+} channels mediated the

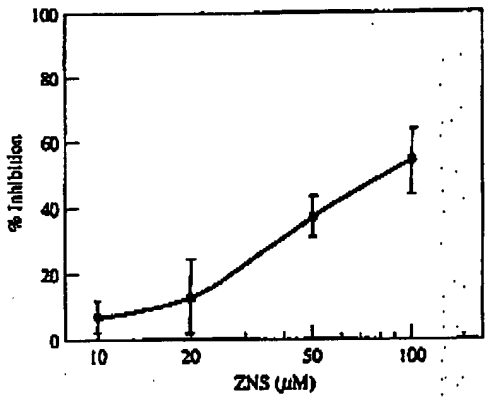


Fig. 2: Plots of ZNS concentration (logarithmic scale) vs. percentage inhibition of T-type I_{Ca} (error bars show mean ± SEM of the number of cells per point). The percent inhibition was calculated for the transient inward current (measured at the peak) elicited by a 0 mV command. Since 0.5% DMSO in the bath solution reduced T-type I_{Ca} by 4.3%, the percent inhibition in the plot represents nominal inhibition minus 4.3%. The curve for T-type I_{Ca}, which was fitted to the data by eye, was constructed from the standard concentration-response equation. This equation assumed one-to-one stoichiometry between ZNS concentration and effect on T-type I_{Ca}.

transition between tonic and phasic firing in thalamic neurons. The blockade of T-type I_{Ca} may play a role in the depressant action of such anitiepileptic drugs as phenytoin, valproic acid, and ethosuximide on repetitive neuronal firing, and may thereby contribute to their efficacy in suppressing epileptic discharges[16–18]. Since ethosuximide and valproic acid are antiepileptics, it is thought that the T-type calcium channel is involved in the generation of absence seizures. This study has shown that ZNS suppresses T-type I_{Ca} without altering its inactivation kinetics or voltage dependence. Two different aspects of T-type calcium channel blockade were distinguished. The first aspect was a resting blockade in which ZNS blocked T-type I_{Ca} in a concentration-dependent manner. At a close equivalent to the therapeutic level, ZNS (50 μM) inhibited T-type I_{Ca} by 38.3 ± 5.8%, a result similar to the findings of Suzuki *et al*[4]. The second aspect was that ZNS enhanced steady-state inactivation. ZNS (50 μM) shifted the T-type channel inactivation curve approximately 20 mV to the negative compared with the control.

In conclusion, ZNS inhibited T-type I_{Ca} in a concentration-dependent manner, and it enhanced steady-state inactivation. The blockade of T-type calcium channels by ZNS could suppress an important component of the inward current that underlies epileptiform cellular bursting, thereby inhibiting the spread of seizure activity.

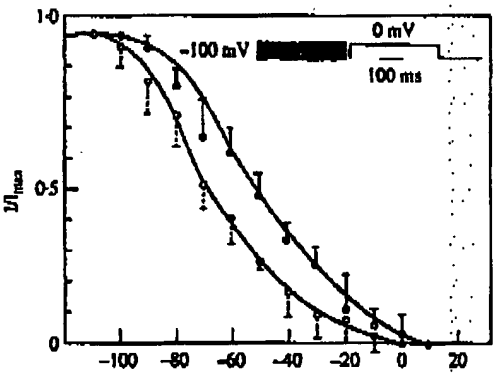


Fig. 3: Voltage-dependence of T-type calcium channel inactivation. The test pulses for the activation of T-type calcium currents (350 ms, 0 mV) were preceded by 350-ms conditioning pulses to various membrane potentials (inset). Current amplitudes, normalized to the value obtained with a test pulse of 0 mv, were plotted as a function of conditioning potential. The application of 50 μM ZNS produced a hyperpolarizing shift of about 20 mV in the T-type calcium channel inactivation curve.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Japan Epilepsy Research Foundation and a Research Grant (4A-1) for Nervous and Mental Disorders from the Ministry of Health and Welfare of Japan.

REFERENCES

1. Seino, M., Miyazaki, H. and Ito, T. Zonisamide. In: (Eds F. Pisani, E. Perruca, G. Avanzini, A. Richens). *New Antiepileptic Drugs. Epilepsy Research* (Suppl. 3). Amsterdam, Elsevier, 1991: 169–174.
2. Rock, D.M., Macdonald, R.L. and Taylor, C.P. Blockade of sustained repetitive action potentials in cultured spinal cord neurons by zonisamide (AD 810, CI 912), a novel anticonvulsant. *Epilepsy Research* 1989; 3: 138–143.
3. Schauf, D.L. Zonisamide enhances slow sodium inactivation in Myxicola. *Brain Research* 1987; 413: 185–188.
4. Suzuki, S., Kawakami, K., Nishimura, S. *et al.* Zonisamide blocks T-type calcium channel in cultured neurons of rat cerebral cortex. *Epilepsy Research* 1992; 12: 21–27.
5. Kito, M., Maehara, M. and Watanabe, K. Antiepileptic drugs-calcium current interaction in cultured human neuroblastoma cells. *Seizure* 1994; 3: 141–149.
6. Miyake, S., Shimo, Y. and Kitamura, T. Characteristics of continuous and functional cell line NB-1, derived from a human neuroblastoma. *Autonomic Nervous System* 1973; 10: 115–120.
7. Hamil, O.P., Marty, A., Neher, E., Sackmann, B. and Sigworth, F.J. Improved patch clamp techniques for

high-resolution current recording from cells and cell-free membrane patches. *Pflüger's Archiv: European Journal of Physiology* 1984; 391: 85–100.

8. Fox, A.P., Nowycky, M.C. and Tsien, R.W. Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurons. *Journal of Physiology (Lond.)* 1987; 394: 149–172.
9. Rogawski, M. and Porter, R.J. Antiepileptic drugs: pharmacological mechanisms and clinical efficacy with consideration of promising developmental stage compounds. *Pharmacological Reviews* 1990; 42: 223–286.
10. Nowycky, M.C., Fox, A.P. and Tsien, R.W. Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature (Lond.)* 1985; 316: 440–443.
11. Kasai, H., Aosaki, T. and Fukuda, J., Presynaptic Ca-antagonist ω-conotoxin irreversibly blocks N-type Ca-channels in chick sensory neurons. *Neuroscience Research* 1987; 4: 228–235.
12. Nowycky, M.C., Fox, A.P. and Tsien, R.W. Long-opening mode of gating of neuronal calcium channels and its promotion by the dihydropyridine calcium agonist Bay K 8644. *Proceedings of the National Academy of Sciences of The United States of America* 1985; 82: 2178–2182.
13. Kito, M., Maehara, M. and Watanabe, K. Three types of Voltage-dependent calcium currents in cultures human neuroblastoma cells. *Nagoya Journal of Medical Science* 1995; 58: 29–33.
14. Miller, R.J. Multiple calcium channels and neuronal function. *Science* 1987; 235: 46–52.
15. Suzuki, S. and Rogawski, M.A. T-type calcium channels mediate the transition between tonic and phasic firing in thalamic neurons. *Proceedings of the National Academy of Sciences of The United States of America* 1989; 86: 7228–7232.
16. Twombly, D.A., Yoshii, M. and Narahashi, T. Mechanisms of calcium channel block by phenytoin. *Journal of Pharmacology and Experimental Therapeutics* 1989; 246: 189–195.
17. Kelly, K.M., Gross, R.A. and Macdonald, R.L. Valproic acid selectively reduces the low-threshold (T) calcium current in rat nodose neurons. *Neuroscience Letters* 1990; 116: 233–238.
18. Coulter, D.A., Huguenard, J.R. and Prince, D.A. Characterization of ethosuximide reduction of low-threshold calcium current in thalamic neurons. *Annals of Neurology* 1989; 25: 582–593.

0013-7227/97/$03.00/0
Endocrinology

Vol. 138, No. 9
Printed in U.S.A.

T-Type Calcium Channels Facilitate Insulin Secretion by Enhancing General Excitability in the Insulin-Secreting β-Cell Line, INS-1*

ARIN BHATTACHARJEE, RICHARD M. WHITEHURST JR., MIN ZHANG, LIN WANG, AND MING LI

Department of Pharmacology (A.B., M.Z., L.W., M.L.) and Department of Pediatrics (R.M.W.), University of South Alabama College of Medicine, Mobile, Alabama 36688

ABSTRACT

The present study addresses the function of T-type voltage-gated calcium channels in insulin-secreting cells. We used whole-cell voltage and current recordings, capacitance measurements, and RIA techniques to determine the contribution of T-type calcium channels in modulation of electrical activity and in stimulus-secretion coupling in a rat insulin secreting cell line, INS-1. By employing a double pulse protocol in the current-clamp mode, we found that activation of T-type calcium channels provided a low threshold depolarizing potential that decreased the latency of onset of action potentials and furthermore increased the frequency of action potentials, both of which are abolished by administration of nickel chloride ($NiCl_2$), a selective T-type calcium channel blocker. Moreover application of high frequency stimulation, as compared with low frequency stimulation, caused a greater change in membrane capacitance (ΔCm), suggesting higher insulin secretion. We demonstrated that glucose stimulated insulin secretion in INS-1 is reduced dose dependently by $NiCl_2$. We conclude that T-type calcium channels facilitate insulin secretion by enhancing the general excitability of these cells. In light of the pathological effects of both hypo and hyperinsulinemia, the T-type calcium channel may be a therapeutic target. (*Endocrinology* 138: 3735–3740, 1997)

GLUCOSE-INDUCED electrical activity in pancreatic β-cells is thought to involve slow waves of membrane depolarization on which action potentials are superimposed (1). Plateau depolarization is initiated via blockade of the ATP-sensitive potassium channels causing trains of calcium-dependent, tetrodotoxin-insensitive action potentials (2). This has been the hallmark of electrical activity in mouse pancreatic β-cells. The trains of action potentials represent the opening and closing of the voltage-dependent L-type calcium channels, major calcium signaling channels also found in neurons and muscle cells. The secretion of insulin has been shown to be dependent on increases in intracellular calcium concentration (3–6), and it has been proposed that the metabolism of glucose will cause depolarization of the cell to membrane voltages that will activate the L-type calcium channels and, therefore, facilitate insulin secretion. Recently, however, it was demonstrated that the electrical activities in human pancreatic β-cells are much more complex because of the prominence of additional inward voltage-dependent ionic currents, namely sodium and T-type calcium currents (7).

The roles of low voltage-activated T-type calcium channels in insulin stimulus-secretion coupling have not been properly assessed, in part because these channels are not normally expressed in the mouse model. A slow deactivating calcium current in rat pancreatic β-cells was reported (8, 9) and more recently the presence of T-type calcium channels was demonstrated in human β-cells (10, 11). The low voltage activation of these calcium channels suggests that they may play a pacemaker role (12). T-type calcium currents have also been shown to trigger burst firing and increase the number and/or frequency of action potentials in neurons (12–14).

In the present study we have investigated the roles of T-type calcium currents in stimulus-secretion coupling in INS-1 cells by using conventional whole-cell as well as perforated whole-cell, patch clamp techniques. INS-1 is a stable rat β-cell line that responds to glucose at a physiological range of concentrations (15–17). INS-1 cells express a considerable level of L-type calcium current as well as T-type calcium current. Because human β-cells demonstrate similar calcium current profiles, the INS-1 cell line then becomes a suitable model for β-cell electrophysiology studies. In this communication, we tested the hypothesis that the T-type calcium current participates in the generation of rhythmic activity and facilitates bursting of action potentials in INS-1 and, therefore, the T-type calcium current is an enhancer of insulin secretion.

Materials and Methods

RIA

The assay is based on the competition between unlabeled insulin and a fixed amount of ^{125}I-labeled insulin for a limited number of insulin-antibody (LINCO Research Inc., St. Charles, MO) binding sites (18). With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added nonradioactive ligand. The separation of antibody-insulin and free insulin was achieved with centrifugation (3000 rpm, 20 min) and decantation of the supernatant. Measurement of the radioactivity in the pellet enabled the amount of labeled insulin in the bound fraction to be calculated. The concentration of unlabeled insulin

Received February 6, 1997.
Address all correspondence and requests for reprints to: Ming Li, Ph.D. Department of Pharmacology, University of South Alabama, College of Medicine Mobile, Alabama 36688. E-mail: mli@jaguar1.usouthal.edu.
* This work was supported by The American Diabetes Association and American Heart Association.

in the sample was then determined by interpolation from a standard curve.

Cell Culture

INS-1 cells were kindly provided by Dr. Barbara E. Corkey (Boston University). This cell line was cultured in RPMI 1640 medium containing 10% FBS, 25 U/ml penicillin, 25 mg/ml streptomycin and 50 μM mercaptoethanol (15) in an atmosphere of 5% CO_2 in air, at 37 C, for 2–5 days before recording. HIT-T15 cells were cultured under identical conditions except media was not supplemented with mercaptoethanol.

Electrical recordings and data analysis

Whole-cell voltage-clamp, current-clamp and capacitance measurements were carried out by the standard giga-seal patch clamp method (19). The whole-cell recording pipettes were made of hemo capillary tubes (Fisher Scientific, Pittsburgh, PA), pulled by a two-stage puller (P-30, Sutter Instrument, Novato, CA) and heat-polished before use. Capacitance measurement recording pipettes were coated with Sylgard (Dow Corning, Midland, MI), with the dipping-in method as previously described (20). Pipette resistance was in the range of 2–5 MΩ in our internal solutions. The voltage-clamp and current-clamp recordings were performed at room temperature (22–25 C), while capacitance measurements were performed at more physiological temperatures (32–35 C). An EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) filtered at 2.9 kHz was used, and data were acquired using Pulse/PulseFit software (HEKA). Voltage-dependent currents have been corrected for linear leak and residual capacitance by using an on-line P/n subtraction paradigm.

Solutions for recording

For whole-cell voltage-clamp recordings, the extracellular solution contained (in mM): 10 $CaCl_2$, 110 tetraethylammonium Cl (TEA-Cl), 10 CsCl, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 40 sucrose, 0.5 3,4-diaminopyridine, at pH 7.3. For steady-state inactivation experiments, a bath solution containing (in mM) 2 $CaCl_2$, 120 NaCl, 15 HEPES, 30 TEA-Cl, and 1 μM tetrodotoxin (TTX) was used. The intracellular solution contained (in mM): 130 *N*-methyl-D-glucamine, 20 EGTA (free acid), 5 bis(2-aminophenoxy)ethane-*N*, *N*,*N'*,*N'*-tetraacetate (BAPTA), 10 HEPES, 6 $MgCl_2$, 4 $Ca(OH)_2$, with pH adjusted to 7.4 with methanesulfonate. A concentration of 2 mM ATP was included in the intracellular solution to prevent run-down of Ca^{2+} currents. For current-clamp recordings, the perforated-patch pipette solution contained (in mM): 140 KCl, 10 NaCl, 2 $MgCl_2$, 5 HEPES, and 0.24 mg/ml amphotericin B, pH 7.4. The bath solution contained 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, and 30 sucrose, pH 7.3. For capacitance measurements, the pipette solution contained 130 Cs-aspartate, 30 HEPES, 2 $MgCl_2$, 10 CsCl, pH 7.4. The bath solution contained 110 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, and 40 sucrose, pH 7.4.

Results

Two types of voltage-dependent calcium currents have been recorded in INS-1 cells as shown in a representative family of calcium currents in an INS-1 cell (Fig. 1A). The cell was held at −80 mV and test potentials, with duration of 200 msec, were applied from −50 to 0 mV. The traces show the presence of both low voltage-activated (T-type) and high voltage-activated (L-type) calcium currents. INS-1 cells consistently exhibited T-type calcium current.

As a low voltage-activated channel, the voltages that are required for both activation and inactivation of the T-type calcium channel are more negative than that of high voltage-activated calcium channels. To determine the possibility that T-channels are in the noninactivated state at cell resting membrane potentials, we characterized the steady-state inactivation of the T-type calcium currents in INS-1 cells (Fig. 1B). Representative cells (n = 3) were given varied prepulse potentials (−90 to −40 mV) of 1000 msec duration then

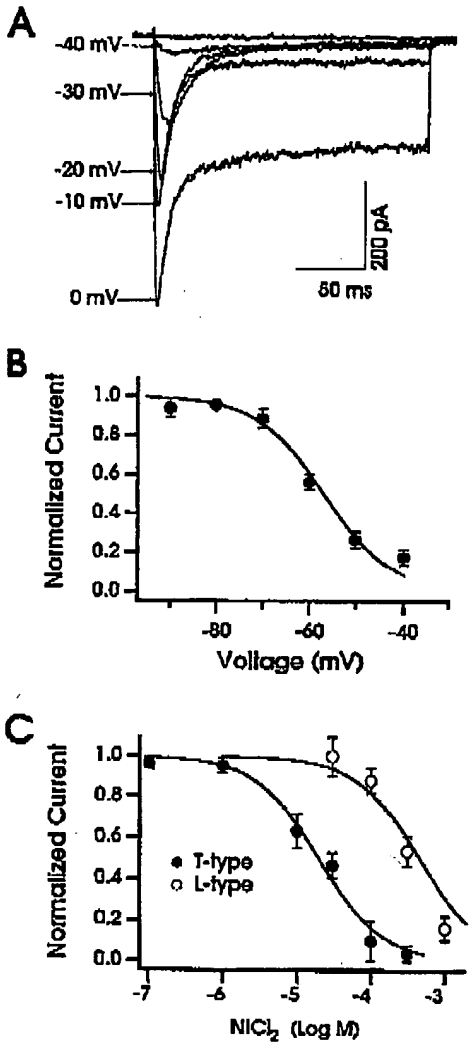


FIG. 1. A, Representative family of calcium currents in an INS-1 cell. B, Plot of steady-state inactivation of calcium currents from INS-1 cells. The obtained currents at this test pulse were then normalized to the maximal current and plotted. C, The inhibitory effect of $NiCl_2$ on T-type calcium currents in INS-1 cells (n = 4). Concentration dependent block of T-type and L-type currents were normalized to the maximal currents. *Error bars* represent SEM.

immediately stepped to a test potential of −30 mV for 100 msec in bath solution containing 2 mM $CaCl_2$ to negate surface potential effects. Currents obtained at this test pulse were then normalized and plotted. A test potential of −30 mV was chosen because the predominant calcium current is T-type. Note that at potentials negative to −65 mV, approximately 90% of the T-type calcium channels are activated whereas at −50 mV less than 40% are activated. This sharp and less negative steady-state inactivation resembles the inactivation properties of T-type calcium current in other endocrine cells (21, 22).

It has been shown that nickel blocks T-type calcium channels in pancreatic β-cells and in other cell types (12, 23–25). The ability of $NiCl_2$ to selectively antagonize T-type calcium

currents *vs.* L-type calcium currents was also examined in INS-1 cells (Fig. 1C) in a dose-dependent fashion. Increasing concentrations of $NiCl_2$ were perfused into the recording chamber and the current amplitudes were measured. For testing the effects of $NiCl_2$ on T-type calcium current, the cells were held at −80 mV with a test pulse applied at −30 mV. To test the effects of $NiCl_2$ on L-type current, the cells were held at −40 mV with a test pulse applied at +10 mV because the calcium current at this voltage comprises mainly of L-type calcium current. Nickel chloride is a very selective blocker of T-type calcium current ($IC_{50} \approx 20$ μM) as much higher concentrations of $NiCl_2$ are required to block L-type calcium current.

We determined the role of T-type calcium current on INS-1 cell electrical activity, using the perforated variant of whole cell current-clamp technique. By applying preceding hyperpolarizing current pulses, we obtained slow rising and falling triangle-like potentials, or low threshold spikes (LTS) (Fig. 2). The amplitude and rising rate of the LTS were increased as the preceding hyperpolarization was increased. In this set of experiments, the extracellular solution contained 2 μM tetradotoxin and 10 μM of nifedipine to block voltage-sensitive sodium channels and L-type calcium channel, respectively. The effective hyperpolarizing voltages ranged from −60 mV to −80 mV, corresponding to the steady-state inactivation potentials of T-type calcium channel; therefore, this LTS is most likely the result of the deinactivation of T-type calcium channel in the cells.

The function of the LTS may be to provide a pacemaker-like intermediate potential that triggers action potential generated by activating sodium channels and/or L-type calcium channels. To test the effects of the LTS on action potential generation, we used a double pulse protocol (Fig. 3). This protocol consists of two identical depolarizing test pulses with an intervening hyperpolarizing pulse, preceding the second test pulse. The first test pulse generates a control potential, and the second test pulse generates a similar control potential plus a new component of membrane potential attributed to T-type calcium channel deinactivation produced by the hyperpolarizing pulse. To eliminate the time-dependent variation of membrane potential during the current clamp recording, the double pulse protocol was employed to ensure an internal control for every voltage recording sweep. The current was clamped in the cell such that the membrane potential remained between −50 to −60 mV. At these potentials, a large proportion of T-type calcium channels should be inactivated. For example, a 1 pA test pulse is not enough to generate an action potential in pulse one, but when the cell is hyperpolarized, an equivalent 1 pA test pulse elicited an action potential in pulse two (Fig. 3). A hyperpolarization to a membrane potential near −80 mV should deactivate all the T-type calcium channels (Fig. 1B).

The effect of the LTS on action potentials was examined directly with the double pulse protocol. This time a test pulse was administered sufficient to generate at least one action potential in the first pulse (Fig. 4A). The number of action potentials after hyperpolarization is greater in the second test pulse. The more depolarized membrane potential due to the LTS may facilitate the initiation of action potentials.

It was also observed that the time it takes to generate the first action potential in the first test pulse was longer than that in the second test pulse. This time was defined as latency of onset of action potential and pooled data (n = 40) from eight cells determined that the latency was greater in test pulse one than test pulse two (Fig. 4B). These results demonstrate that T-type calcium current decreases the latency of onset of action potential and increases the number of action potentials.

To further establish that T-type calcium current enhances

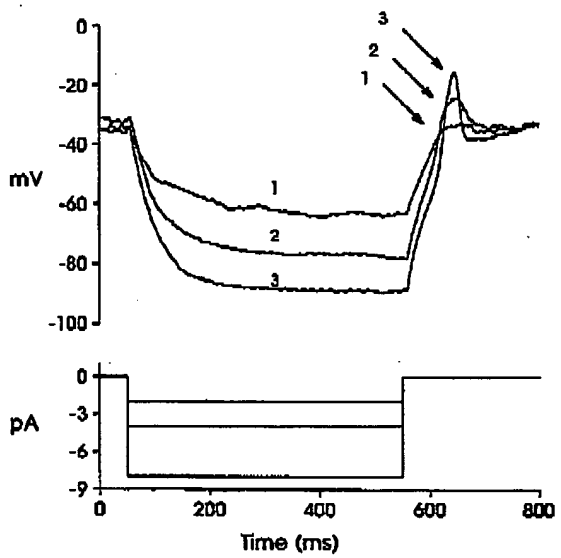


FIG. 2. The generation of LTS in INS-1 cells. Membrane potentials were recorded in current-clamp mode using the perforated-patch variant of whole-cell patch clamp. Preceding hyperpolarizing current pulses, with increasing magnitude (indicated by *arrows*) were applied resulting in slow rising and falling triangle-like potentials, or LTS. The extracellular solution contained 150 mM NaCl, 2 mM $CaCl_2$, 2 μM tetradotoxin, and 10 μM nifedipine.

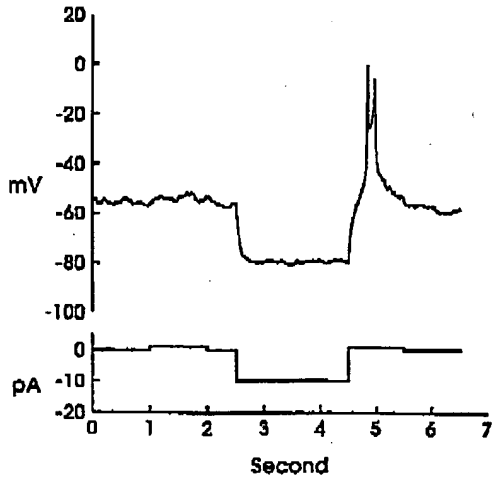


FIG. 3. Representative trace of the double pulse protocol in an INS-1 cell. The current was clamped such that the membrane potential remained between −55 and −60 mV. Both test pulses were of 1 pA magnitude.

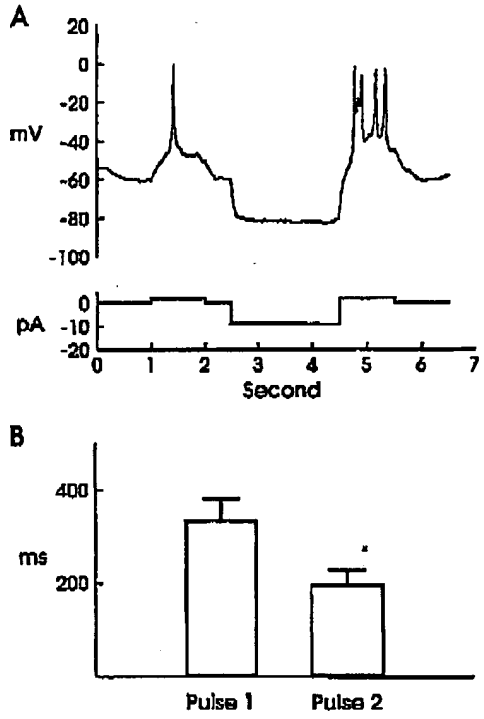


FIG. 4. The LTS increases the frequency of action potentials. A, A current (2 pA) was injected sufficient to generate at least one action potential in the test pulse one. B, Comparison of latencies of onset of action potentials between test pulse one and test pulse two with the double-pulse protocol (8 cells, n = 40 traces). *Error bars* represent SEM. The *asterisk* represents $P \leq 0.01$ with Student's *t* test.

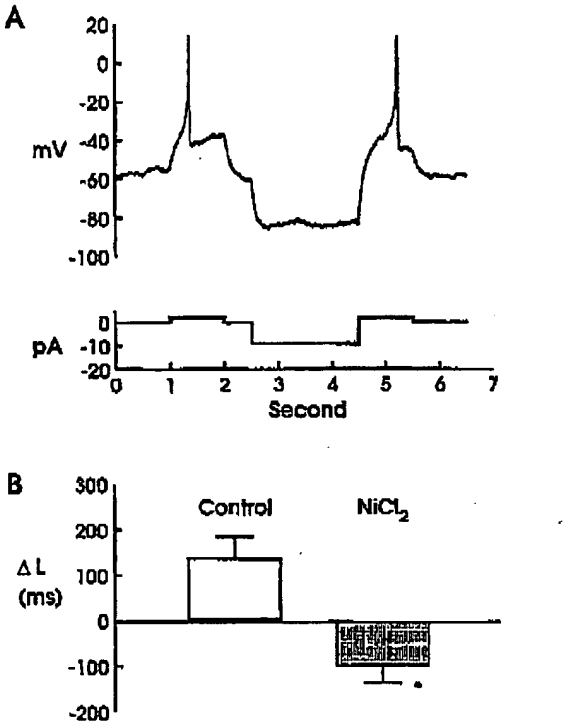


FIG. 5. A, Representative trace where 100 μM nickel is applied to the bath. $NiCl_2$ caused a delay in action potential onset in the second pulse. B, $NiCl_2$ caused a longer latency of action potential onset in test pulse as determined by a negative Δlatency (five cells, n = 20 traces). *Error bars* are SEM. The *asterisk* represents $P \leq 0.01$ with Student's *t* test.

cell excitability, we administered 100 μM $NiCl_2$ to effectively block T-type calcium channels. In contrast to the control experiments, $NiCl_2$ caused a delay in the onset of an action potential in the second test pulse (Fig. 5A) and a decrease in number of action potentials during the second test pulse. The averaged difference between latencies of action potential onset in test pulse one and two, defined as Δ latency, was taken under conditions with and without application of $NiCl_2$. $NiCl_2$ caused an averaged 100 msec greater latency of action potential onset in test pulse two, indicated by a negative Δ latency (Fig. 5B).

The time it takes to charge the membrane capacitor after hyperpolarization may contribute to this negative Δ latency. Theoretically, the time course of the membrane capacitance discharge can be calculated by the following equation (26): $E = E_0 e^{-t/RC}$, where the membrane potential (E) at any moment is inversely proportional to the time (t) of discharging with the time course ($\tau = RC$). For the INS-1 cells, the membrane resistance (R_m) is approximately 3 giga-Ohm; the membrane capacitance (C_m) is averaged 20–25 pF, therefore the τ is estimated to be 60 msec in our experimental conditions. If we assume that the T-type calcium channel activation threshold is approximately −55 mV, the Δ latency for charging the membrane from first pulse (−60 mV) and the second pulse (−80 mV) can be estimated. The estimated Δ latency is between 96 and 120 msec, which fits roughly with our experimental data. The effect of T-type calcium current on latency (Fig. 4B) in our model then, is an underestimate value based on the inherent nature of membrane capacitance.

It has been demonstrated that a single action potential is sufficient to evoke a transient rise in $[Ca^{2+}]_i$ in mouse β-cells (27). Higher frequency of action potentials has been demonstrated to cause a greater and longer rise in $[Ca^{2+}]_i$ and increases in β-cell exocytosis (28). In our system, we measured changes in membrane capacitance in response to two different stimulation frequencies, mimicking the electrical activity in these cells as an index of exocytosis. This uses the fact that exocytosis involves fusion of the secretory granules with the plasma membrane, resulting in an increase in the cell surface area. Cells were held at −60 mV and then repetitive depolarizations to 0 mV, each a duration of 10 msec were applied for 3 sec. The low frequency stimulation consisted of 10 pulses/sec, and the high frequency stimulation consisted of 20 pulses/sec and both stimulations were applied to the same cell (Fig. 6A). High frequency stimulation caused a greater increase in membrane capacitance compared with low frequency stimulation, suggesting that more secretory vesicles fused to the membrane with high frequency stimulation. Pooled data from 12 cells of approximate equal size demonstrated that high frequency stimulation induces a greater change in membrane capacitance than low frequency stimulation (Fig. 6B). This should be expected because high frequency stimulation will lead to higher cy-

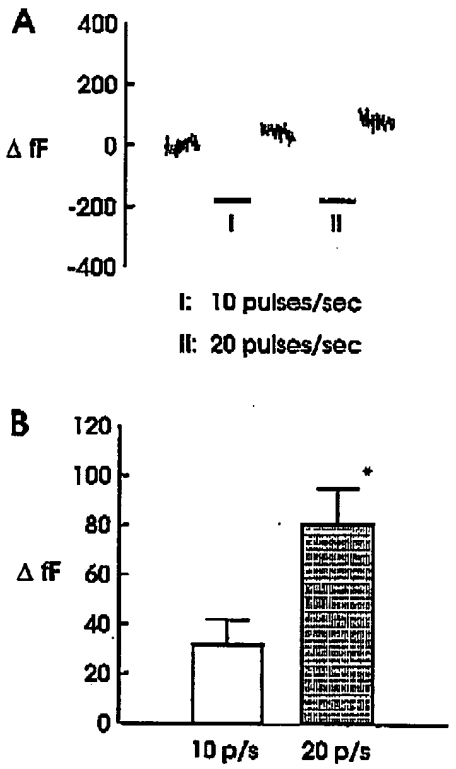


FIG. 6. A, Representative trace of changes in membrane capacitance (one measurement of cell capacitance for every 5 sec) in an INS-1 cell when two different stimulations were applied to the same cell. Cells were held at −60 mV and then repetitive depolarizations to 0 mV, each a duration of 10 msec were applied for every 3 sec. The low frequency stimulation consisted of 10 pulses/sec, and the high frequency stimulation consisted of 20 pulses/sec. B, A high frequency stimulation induces a greater change in membrane capacitance than a low frequency stimulation (n = 12 cells). Data were collected with low frequency pulse applied first and also in the reverse order. *Error bars* are SEM. The *asterisk* represents $P \leq 0.01$ with Student's *t* test.

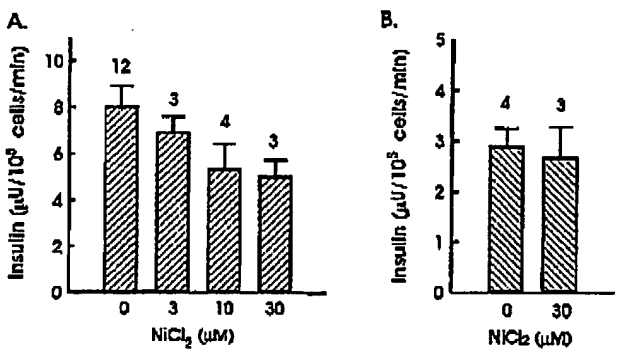


FIG. 7. A, The dose-dependent effect of nickel on insulin secretion in INS-1 cells. Cells were placed in a medium containing 11.1 mM glucose for 1 h with and without $NiCl_2$. B, The effect of 30 μM nickel on HIT-15T cells. Insulin release was determined by a standard RIA. *Numbers above bars* represent the numbers of independent experiments performed. *Error bars* represent SEM.

tosolic calcium concentrations and will likely facilitate more secretory vesicles fusing to the membrane.

To directly demonstrate the role of T-type calcium current in glucose-stimulated insulin secretion, we incubated INS-1 cells with 11.1 mM glucose and 0, 3, 10, or 30 μM $NiCl_2$ and measured the insulin release using a standard RIA (Fig. 7A). $NiCl_2$ reduced insulin secretion in a dose-dependent manner. On the other hand, clonal hamster-secreting cells (HIT-T15), which do not consistently exhibit T-type calcium current (29), were not affected by 30 μM $NiCl_2$ (Fig. 7B). This suggests that T-type calcium current in INS-1 cells significantly contributes to glucose-stimulated insulin secretion.

Discussion

In this study, we have determined a role for T-type calcium channels in stimulus-secretion coupling. The double pulse protocol we employed demonstrated the effects of T-type calcium current in altering the electrical activity of INS-1 cells. We believe that T-type calcium currents have a permissive role and contributes to the depolarization of the membrane, allowing for activation of the L-type calcium channels. The true contribution of T-type calcium current may be seen in the intermittent repolarizing phases of the glucose-induced electrical activity cycle. The low threshold spike may give rise to the plateau potential. Ultimately, T-type calcium channels may play a pacemaker role especially at postabsorptive glucose levels.

The resting membrane potential of INS-1 cells has been reported to be between −72 and −80 mV (17, 30) and 15 mM glucose depolarizes the cell to approximately −56 mV. Based upon the voltage-dependent inactivation curve obtained for T-type calcium channels in INS-1 cells, we can conclude that T-type calcium currents are a significant contributor to the underlying electrical activities of INS-1 cells in the physiological range of glucose concentrations. Indeed, the voltage-dependent inactivation profile for the T-type calcium current in INS-1 cells is very similar to other endocrine cell types, where a very large proportion of the channels are still available for conducting current at voltages negative to −55 mV.

T-type calcium current mediated LTS has been demonstrated to play a critical role in regulating the rhythmicity in the thalamic neuronal network (31). In that system, each action potential is followed by a marked after-hyperpolarization (A-HP), which provides a potential required for deinactivation of T-type calcium channels. In INS-1 cells, however, no such A-HP is seen during the repetitive firing. The function of T-type calcium current may be to provide a small but sustained depolarization which facilitates higher firing frequency.

It has been demonstrated that 100 μM nickel chloride reduces the frequency of action potentials in human β-cells (7). In our experiments, we observed that low concentrations of $NiCl_2$ reduced the number of action potentials in INS-1 cells. The underlying mechanism responsible for this effect is unclear. It is possible that the T-type calcium current in β-cells undergoes voltage-dependent potentiation as is seen in other cell types (32, 33). Further T-type calcium current recruitment upon strong depolarization may facilitate the firing of subsequent action potentials and thus increase cell excitability.

We have only addressed the contribution of T-type calcium current in terms of electrical activity. The amount of

calcium that enters via T-type calcium channels will also be significant for secretion, particularly to vesicles that are in close proximity to the membrane. For example, a calcitonin-secreting cell line has been shown to only possess T-type calcium channels and not L-type calcium channels (21). The T-type calcium channels in this cell type are the major Ca^{2+} signaling channels for secretion. It is conceivable that T-type calcium channels facilitates much of the calcium entry (unpublished observations) at lower glucose concentrations.

It has been shown that T-type calcium current are important for neuronal and cardiac electrical activities (34–40). The functions of T-type calcium channels in endocrine cell types have also been emerging (21, 22, 41, 42). The functions of T-type calcium channels in pancreatic β-cells have largely been overlooked due in part to the choice of β-cell models. It has been well documented that the mouse model does not exhibit T-type calcium current. The INS-1 cell line responds normally to glucose, and the presence of T-type calcium channels may indicate that this model is suitable for electrophysiology studies since T-type calcium channels are prominent in human pancreatic β-cells.

References

1. Ashcroft FM, Rorsman, P 1991 Electrophysiology of the pancreatic β-cell. Prog Biophys Mol Biol 54:87–143
2. Ashcroft FM, Kakei M, Gibson JS, Gray DW, Sutton R 1989 The ATP-sensitive K-channel from human pancreatic β cells. Diabetologia 32:591–598
3. Berggren PO, Larsson O 1994 Ca^{2+} and pancreatic β-cell function. Biochem Soc Trans 22:12–18
4. Hellman B, Gylfe E 1986 Calcium and the control of insulin secretion. In: Cheung WY (eds) Calcium and Cell Function. Academic Press, Orlando, vol VI, pp 253–326
5. Henquin JC, Meissner HP 1984 Significance of ionic fluxes and changes in membrane potential for stimulus-secretion coupling in pancreatic β-cells. Experientia 40:1043 1052
6. Prentki M, Matschinsky FM 1987 Ca^{2+}, cAMP and phospholipid-derived messengers in coupling mechanisms of insulin secretion. Physiol Rev 67:1185–1249
7. Barnett DW, Pressel DM, Misler, S 1995 Voltage-dependent Na^+ and Ca^{2+} currents in human pancreatic islet β cells: evidence for roles in the Ca generation of action potentials and insulin secretion. Pflügers Arch 430:272–282
8. Ashcroft FM, Kelly RP, Smith PA 1990 Two types of Ca^{2+} channels in rat pancreatic β cells. Pflügers Arch 415:504–506
9. Hiriat M, Matteson DR 1988 Na^+ and two types of Ca^{2+} channels in rat pancreatic β-cells identified by reverse hemolytic plaque assay. J Gen Physiol 91:617 639
10. Misler S, Barnett DW, Pressel DM, Gillis KD, Scharp DW, Falke LC 1992 Stimulus secretion coupling in β-cells of transplantable human islets of Langerhans. Evidence for a critical role of Ca^{2+} entry. Diabetes 41:662 670
11. Misler S, Barnett DW, Gillis KD, Pressel DM 1992 Electrophysiology of stimulus secretion coupling in human β-cells. Diabetes 41:1221 1228
12. Hagiwara N, Irisawa H, Kameyama M 1988 Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. J Physiol Lond 395:233–253
13. Christenson J, Hill RH, Bongianni F, Grillner S 1993 Presence of low voltage activated calcium channels distinguishes touch from pressure sensory neurons in the lamprey spinal cord. Brain Res 608:58–66
14. White G, Lovinger DM, Weight FF 1989 Transient low-threshold Ca^{2+} current triggers burst firing through an afterdepolarizing potential in an adult mammalian neuron. Proc Natl Acad Sci USA 86:6802 6806
15. Asfari M, Janjic D, Meda P, Li C, Halban PA 1992 Establishment of 2-mercaptoethanol-dependent differentiated insulin secreting cell lines. Endocrinology 130:167 178
16. Janjic D, Wollheim, CB 1992 Islet cell metabolism is reflected by MTT (tetrazolium) colormetric assay. Diabetologia 35:482–485
17. Ullrich S, Abel KB, Lehr S, Gregor R 1996 Effects of glucose, forskolin and tobutamide on membrane potential and insulin secretion in the insulin secreting cell line INS-1. Pflügers Arch 432:630–636
18. Makulu DR, Vichick D, Wright PH, Sussman KE, Yu FL 1969 Insulin Immunoassay by back titration using alcohol precipitation of insulin antibody complexes. Diabetes 18:660–669
19. Hamill OP, Marty A, Neher, E, Sakmann B, Sigworth FJ 1981 Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391:85–100
20. Li M, McCann JD, Welsh MJ 1990 Apical membrane Cl^- channels in airway epithelia: anion selectivity and effect of an inhibitor. Am J Physiol 259:C295–C301
21. Biagi BA, Mlinar B, Enyeart JJ 1992 Membrane currents in a calcitonin-secreting human C cell line. Am J Physiol 263:C986–C994
22. Bossu JL, De Waard M, Feltz A 1991 Inactivation characteristics reveal two calcium currents in adult bovine chromaffin cells. J Physiol Lond 437:603–620
23. Wang L, Bhattacharjee A, Fu J, Li M 1996 Abnormally expressed low-voltage-activated calcium channels in β-cells from NOD mice and a related clonal cell line. Diabetes 45:1678–1683
24. Fox AP, Nowycky MC, Tsien RW 1987 Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. J Physiol Lond 394:149–172
25. Narahashi T, Tsunoo A, Yoshii M 1987 Characterization of two types of calcium channels in mouse neuroblastoma cells. J Physiol Lond 383:231–249
26. Hille B 1991 In: Ionic Channels of Excitable Membranes. Sinauer, Sunderland, Massachusetts, pp 9–11
27. Rorsman P, Ämmälä C, Berggren P-O, Bokvist K, Larsson O 1992 Cytoplasmic calcium transients due to single action potentials and voltage clamp depolarization in mouse pancreatic B-cells. EMBO J 11:2877 2884
28. Ämmälä C, Eliasson L, Bokvist K, Larsson O, Ashcroft FM, Rorsman P 1993 Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic B-cells. J Physiol Lond 472:665–688
29. Keahey HH, Rajan AS, Boyd AE 3d, Kunze DL 1989 Characterization of voltage dependent Ca^{2+} channels in β cell line. Diabetes 38:188–193
30. Abel K-B, Lehr S, Ullrich S 1996 Adrenaline-, not somatostatin-induced hyperpolarization is accompanied by a sustained inhibition of insulin secretion in INS-1 cells. Activation of sulphonylurea K^+_{ATP} channels is not involved. Pflügers Arch 432:89–96
31. Jahnsen H, Llinás R 1984 Electrophysiological properties of guinea-pig thalamic neurones: an *in vitro* study. J Physiol Lond 349:205–226
32. Publicover SJ, Preston MR, El Haj AJ 1995 Voltage-dependent potentiation of low-voltage activated Ca^{2+} channel currents in cultured rat bone marrow cells. J Physiol Lond 489:649 661
33. Alvarez JL, Rubio LS, Vassort G 1996 Facilitation of T-type calcium current in bullfrog atrial cells:voltage dependent relief of a G protein inhibitory tone. J Physiol Lond 491:321–334
34. Bean BP 1985 Two kinds of calcium channels in canine atrial cells. Differences in kinetics, selective, and pharmacology. J Gen Physiol 86:1–30
35. Carbone E, Lux HD 1984 A low voltage-activated, fully inactivating Ca channel in vertebrate sensory neurones. Nature Lond 310:501–502
36. Deschenes M, Paradis M, Roy JP, Steriade M 1984 Electrophysiology of neurons of lateral thalamic nuclei in cat resting properties and burst discharges. J Neurophysiol 51:1196–1219
37. Llinás RR 1988 The intrinsic electrophysiological properties of mammalian neurons: insights into central nervous system function. Science 242:1654–1664
38. Satoh H 1995 Role of T type Ca^{2+} channel inhibitors in the pacemaker depolarization in rabbit sino-atrial nodal cells. Gen Pharmacol 26:581–587
39. Wilcox KS, Gutnick, MJ, Christoph, GR 1988 Electrophysiological properties of neurons in the lateral habenula nucleus: an *in vitro* study. J Neurophysiol 59:212–225
40. Huguenard JR, Prince DA 1994 Intrathalamic rhythmicity studied *in vitro*: nominal T current modulation causes robust antioscillatory effects. J Neurosci 14:5485–5502
41. Barrett PQ, Ertel EA, Smith MM, Nee JJ, Cohen CJ 1995 Voltage gated calcium currents have two opposing effects on the secretion of aldosterone. Am J Physiol 268:C985–C992
42. Ritchie AK 1993 Estrogen increases low voltage-activated calcium current density in GH3 anterior pituitary cells. Endocrinology 132:1621 1629:1993

Calcium Channel Blockers

Recommended Reading

- Goodman and Gilman (9th ed.), Chapter 32, pp. 767-774 or
- Katzung (7th ed.), pp. 186-191

Supplemental Reading

- "Molecular Biology of Calcium Channels in the Cardiovascular System", A.M. Katz, Am. J. Cardiol. 80(9A) 17I-22I (1997)

Objectives

To understand: 1) the role of Ca^{++} in cardiovascular function; 2) the basic principles of Ca^{++} homeostasis; 3) the pharmacology of Ca^{++}-channel blockers; 4) the therapeutic applications of Ca^{++}-channel blockers.

Outline of Topics

- Introduction
  - Role of Ca^{++} in Cardiac and Smooth Muscle
  - Regulation of Intracellular (Cytoplasmic) Ca^{++} Concentration
  - Molecular Structures Involved in Ca^{++} Regulation
  - Sites of Ca^{++}-Channel Blocker Action
  - History of Ca^{++} Channel Blockers
- Chemistry of Ca^{++} Channel Blockers
- Pharmacology of Ca^{++} Channel Blockers
  - Effects on Vascular Smooth Muscle
  - Effects on Cardiac Cells
  - Hemodynamic Effects
  - Drug-Specific Effects
  - Pharmacokinetics
  - Toxicities and Side Effects
- Therapeutic Uses of Ca^{++} Channel Blockers
  - Angina
  - Arrhythmias
  - Hypertension
  - Less Common/Experimental Uses of Calcium Antagonists

Introduction

Role of Ca^{++} in Cardiac and Smooth Muscle

- Changes in intracellular Ca^{++} regulate contraction through different mechanisms in cardiac and smooth muscle:
  - In cardiac muscle, Ca^{++} binding to troponin C relieves troponin inhibition of actin-myosin interactions
  - In smooth muscle, Ca^{++} binding to calmodulin activates myosin light chain kinase which in turn phosphorylates the P-light chain of myosin. This triggers contraction (i.e.- actin-myosin interactions), but there appear to be additional Ca^{++} regulatory mechanisms

Regulation of Intracellular (Cytoplasmic) Ca^{++} Concentration

- There are a variety of ion pumps, channels, and exchangers that are directly involved in controlling intracellular Ca^{++}, thus many possible sites for therapeutic agents to act
- At rest, the cytoplasmic free Ca^{++} concentrations are normally maintained at very low concentrations (<100 nM) relative to the Ca^{++} concentrations found extracellularly (> 1 mM)
- Upon stimulation, the free Ca^{++} concentration in the cytoplasm can be rapidly elevated to concentrations > 1 uM; this increase occurs through the opening of channels in the sarcolemma/plasmalemma and sarcoplasmic/endoplasmic reticulum
- When stimulation is removed, ATP-dependent ion-pumps and Na^+/Ca^{++} exchangers return the cytoplasmic free Ca^{++} concentrations to resting levels

Molecular Structures Involved in Ca^{++} Regulation

- Molecular structures in the plasmalemma/sarcolemma that regulate Ca^{++} influx:
  - Plasmalemmal Ca^{++}-channels allow extracellular Ca^{++} to enter cells and fall into 3 major categories: voltage-dependent, receptor-operated, and stretch-operated
    - **Voltage (potential)-dependent Ca^{++}-channels** (homologous to Na^+- and K^+-channels, they consist of at least three types in the body: L, T, and N, and possibly a P-type)
      - **L-type channels**: Large sustained conductance, inactivate slowly, widespread in cardiovascular system, are responsible for plateau phase (slow inward current) of action potential, may trigger release of internal Ca^{++}, sensitive to Ca^{++}-channel blockers, cardiac L-channels are regulated by cAMP-dependent protein kinase (phosphorylation enhances probability of channel opening at a given membrane potential)
      - **T-type channels**: Structurally similar to L-type channels; inactivate rapidly; highest abundance in cardiac cells that lack a T-tubule system (SA nodal tissue); involved in cardaic pacemaker activity, growth regulation, and triggering contraction in vascular smooth muscle; low abundance in adult ventricular myocardium
        - T-type channels are not very sensitive to most of the L-type Ca^{++}-channel blockers (mibefradil being the exception)
      - **N-type channels**: Appear to be found only in neuronal cells, and are not very sensitive to the Ca^{++}-channel blockers used for treating cardiovascular disorders
    - **Receptor-operated Ca^{++}-channels** (e.g. $alpha_1$-adrenergic receptors): Not very sensitive to Ca^{++}-channel blockers

        - **"Stretch"-operated or "leaky" Ca^{++}-channels** (may be important in maintaining vascular smooth muscle tone): Do not appear to be sensitive to Ca^{++}-channel blockers
- Molecular structures in the plasmalemma responsible for Ca^{++} efflux:
    - **Ca^{++}-pumps (ATPases)**: Actively extrude Ca^{++} against a large gradient; some forms of this enzyme are calmodulin-regulated
    - **Na^+/Ca^{++}-exchanger ($3Na^+$ / $1Ca^{++}$)**: A major mechanism for removal of cytoplasmic calcium in myocardium; rate of Ca^{++} efflux depends upon Na^+ gradient across plasmalemma
- Intracellular molecular structures involved in regulating cytoplasmic Ca^{++}:
    - Molecular structures in the sarcoplasmic/endoplasmic reticulum (SR), an important intracellular site for sequestration and rapid release of Ca^{++}
        - **Ca^{++} release channels**: IP_3-sensitive, Ca^{++}-sensitive, ryanodine-sensitive
        - **Ca^{++}-ATPases (pumps)**: Responsible for resequestration of Ca^{++}
- Molecular structures in the mitochondria, a slowly exchanging Ca^{++} reservoir
    - **Ca^{++} uniporter**: Ca^{++} uptake is driven by proton extrusion
    - **Electroneutral Na^+/Ca^{++} exchanger**

Sites of Ca^{++}-Channel Blocker Action

- The conventional channel blockers bind to L-type channels ("slow channels") which are abundant in cardiac and smooth muscle (which may partially explain rather selective effects on the cardiovascular system)
    - Different classes of L-type Ca^{++} channel blockers bind to different sites on the $alpha_1$-subunit which is also the major channel-forming subunit ($alpha_2$, beta, gamma, delta subunits are also present)
    - Different sub-classes of L-type channel exist which may contribute to tissue selectivity
- Newer calcium channel blockers with different specificities have also been developed:
    - Bepridil, a drug with Na^+ and K^+ channel blocking activites in addition to L-type channel blocking activities
    - Mibefradil, a drug with T-type calcium channel blocking activity as well as L-type calcium channel blocking activity

History of Ca^{++} Channel Blockers

- 1962 - Verapamil reported to possess negative inotropic & chronotropic effects not seen with other vasodilatory agents such as nitroglycerin (Hass &Hartfelder)
- 1967 - Fleckenstein et al. suggest that verapamil's negative inotropic effect involved reduction of Ca^{++} movement into cardiac myocytes
- 1972 - Kohlhardt et al. show that D600, a verapamil derivative, blocks Ca^{++} flux through the slow channel
- 1987 - Sales of calcium channel blockers in U.S. approx. $700 million
- 1992 - Sales of calcium channel blockers in U.S. approx. $3 billion
- 1992-1993 - Nifedipine (Procardia), diltiazem (Cardizem), and verapamil (Calan) were among the top 20 most widely sold name brand drugs in the U.S.

Chemistry of Ca^{++} Channel Blockers

- Five major classes of Ca^{++} channel blockers are known with diverse chemical structures:

1. Benzothiazepines: Diltiazem
2. Dihydropyridines: Nicardipine, nifedipine, nimodipine and many others
    - There are also dihydropyridine Ca^{++}-channel *activators* (Bay K 8644, S 202 791)
3. Phenylalkylamines: Verapamil
4. Diarylaminopropylamine ethers: Bepridil
5. Benzimidazole-substituted tetralines: Mibefradil

Pharmacology of Ca^{++} Channel Blockers

Effects on Vascular Smooth Muscle

- L-type Ca^{++} channel blockers inhibit L-type voltage-dependent Ca^{++} channels
- T-type Ca^{++} channel blockers inhibit T-type voltage-dependent Ca^{++} channels
- Little or no effect of Ca^{++} channel blockers on receptor-operated channels or on release of Ca^{++} from SR
- "Vascular selectivity" is seen with the Ca^{++} channel blockers
    - Decreased intracellular Ca^{++} in arterial smooth muscle results in relaxation (vasodilatation) -> decreased cardiac afterload (aortic pressure)
    - Little or no effect of Ca^{++}-channel blockers on venous beds -> no effect on cardiac preload (ventricular filling pressure)
    - Specific dihydropyridines may exhibit greater potencies in some vascular beds (e.g.- nimodipine more selective for cerebral blood vessels, nicardipine for coronary vessels)
    - Little or no effect on nonvascular smooth muscle (e.g. -tracheal smooth muscle)

Effects on Cardiac Cells

Magnitude and pattern of cardiac effects depend on the class of Ca^{++} channel blocker (see Table 1)

- Negative inotropic effects are seen with some of the L-type channel blockers (a direct effect on myocardial L-type channels):
    - The negative inotropic effect is due to reduced inward movement of Ca^{++} during the action potential plateau phase (due to inhibition of slow (L-type) channel)
    - Dihydropyridines have very modest negative inotropic effects
    - Mibefradil (a T-type channel blocker) has no negative inotropic effects because there appear to be few T-type channels in adult ventricular muscle
- Negative chronotropic/dromotropic effects (pacemaker activity/conduction velocity) are also seen with some of the Ca^{++} channel blockers
    - Verapamil (and to a lesser extent diltiazem) decrease the rate of recovery of the slow channel in AV conduction system and SA node, and therefore act directly to depress SA node pacemaker activity and slow conduction
        - Ca^{++}-channel block by verapamil and diltiazem is frequency- and voltage-dependent, making them more effective in cells that are rapidly depolarizing
    - Mibefradil has negative chronotropic and dromotropic effects

        - T-type channels are important for regulating Ca^{++} influx in pacemaker cells and cells of the conduction system
    - Nifedipine and related dihydropyridines do not have significant direct effects on the atrioventricular conduction system or sinoatrial node at normal doses, and therefore do not have *direct* effects on conduction or automaticity
        - The dihydropyridines can cause reflex increases in heart rate because of their potent vasodilating effects

Hemodynamic Effects

- All of the clinically-approved Ca^{++}-channel blockers:
    - Decrease coronary vascular resistance and increase coronary blood flow
    - Decrease peripheral resistance via vasodilatation of arterioles
    - Are without significant effect on venous tone at normal doses

Drug-Specific Effects (also see Table 1)

- Dihydropyridines (e.g. nifedipine, nicardipine, and nimodipine)
    - Vasodilatation of arterial resistance vessels causes a reflex increase in sympathetic response
        - Because the dihydropyridines have very weak effects on the SA node and AV junction, there is an increase in heart rate due to the increase in sympathetic tone
        - Any weak direct negative inotropic effect of the drug is overwhelmed by the strong reflex sympathetic response
    - The overall hemodynamic effect is a drop in blood pressure, an increase in heart rate and contractility, and an increase in cardiac output
- Verapamil
    - At doses that cause peripheral vasodilatation, verapamil has greater direct negative chronotropic, dromotropic (conduction), and inotropic effects than the dihydropyridines
    - The drug's direct negative chronotropic and dromotropic effects are able to overcome any reflex sympathetic response to the lowering of blood pressure, resulting in a drop in heart rate
    - The drug's direct negative inotropic effects can also overcome the reflex sympathetic response, resulting in a lowering of myocardial contractility
    - In patients with left ventricular dysfunction where sympathetic tone may already be high, the drug can can cause a dangerous decrease in contractility
- Diltiazem
    - The hemodynamic effects of diltiazem are intermediate between the dihydropyridines and verapamil
    - The drug causes a modest lowering of heart rate and modest decrease in myocardial contractility, both of which are less than verapamil for a given drop in blood pressure
- Mibrefradil
    - This agent is a potent peripheral and coronary vasodilator. Its chronic effects on blood pressure, heart rate, and cardiac conduction velocity (PQ interval) are comparable to those of verapamil and diltiazem
    - In contrast to verapamil and diltiazem, this agent appears to have negligible negative inotropic effects
    - In contrast to the dihydropyridines, reflex tachycardia does not occur with mibefradil
    - This drug was voluntarily withdrawn from the market by Roche on June 8, 1998 less than one year after its introduction due to interactions with a variety of commonly used drugs

Table 1. Relative Cardiovascular Effects of Prototypical Calcium Channel Blockers (adapted from Goodman and Gilman (9th ed.) and Massie, Am. J. Cardiol. 80(9A)23I-32I(1997)).

Compound	Coronary Vasodilation	Suppresion of Cardiac Contractility	Suppresion of SA Node	Suppression of AV Node
Verapamil	++++	++++	+++++	+++++
Diltiazem	+++	++	+++++	++++
Nifedipine	+++++	+	+	0
Nicardipine	+++++	0	+	0
Bepridil	(+++)	(+++)	(+++++)	(++++)
Mibefradil	(++++)	(0)	(++++)	(++++)

The relative effects are ranked from no effect (0) to most prominent (+++++). Effects indicated in parentheses are estimated effects.

Pharmacokinetics

- All clinically-approved compounds are available for oral administration
  - Verapamil is also available for i.v. administration for interrupting supraventricular arrhythmias
- Absorption is nearly complete after oral administration
- Bioavailability is reduced because of first-pass hepatic metabolism
- There is significant binding of all channel blockers to plasma proteins (70-99%)
- Therapeutic effects are evident within 30-60 min after oral dose; peak effects within 15 min i.v.
- Typical plasma half-life is 1.5 to 6 hours (24-40 hours for bepridil, some of the newer dihydropyridines, and mibefradil)
  - Half-lifes may increase with repeated oral dose due to hepatic saturation
  - Longer half-lifes for elderly patients and patients with hepatic cirrhosis or renal insufficiency
- Verapamil, diltiazem, and possibly nicardipine inhibit hepatic enzymes
- Metabolism of verapamil, diltiazem, and nifedipine is inducible
- Diltiazem and verapamil have vasodilatory metabolites; dihydropyridines do not

Toxicities and Side Effects

- The calcium channel blockers are generally well-tolerated
- The most common side effects, particularly with dihydropyridines, are due to excessive vasodilatation (i.e.- dizziness, hypotension, headache, flushing, edema, etc.)
- Aggravation of myocardial ischemia has been reported, perhaps due to excessive hypotension resulting in decreased coronary perfusion, selective vasodilation in non-ischemic regions ("coronary steal"), and increased oxygen demand due to reflex tachycardia
  - Because of lower capacity to induce excessive peripheral arterial dilation and reflex tachycardia, verapamil and diltiazem are less likely to aggravate myocardial ischemia
- Serious toxic effects (bradycardia, transient asystole, exacerbation of heart failure) are rare and usually occur under specific conditions:
  - After i.v. administration of verapamil
  - During concurrent channel blocker/beta-adrenergic blocker administration

- **i.v. verapamil is specifically contraindicated in combination with beta-blockers due to the possibility of AV block or severe depression of ventricular function**
- Patients with moderate to severe ventricular dysfunction, SA node or AV conduction disturbances, and systolic blood pressures below 90 mm Hg should not be treated with verapamil or diltiazem

- Some channel blockers (e.g. - verapamil) can cause an increase in plasma digoxin levels and are therefore contraindicated for use in treating digitalis toxicity; AV block can also occur with concurrent treatment with channel blockers and digitalis
- Though mibefradil was found to be safe and well-tolerated when used alone, it inhibits the P-450 enzyme CYP 3A4 and thus can interfer with the metabolism of at least 26 other drugs, including certain HMG-CoA reductase inhibitors and other calcium channel blockers. When used in combination with other heart-rate lowering drugs such as beta-blockers, severe bradycardia could occur. Due to the difficulties in avoid these serious drug interactions, mibefradil was thus withdrawn from the market .

Therapeutic Uses of Ca^{++}-Channel blockers

The primary indications for the Ca^{++}-channel blockers are angina, arrhythmias, and hypertension:

Angina (also see Antianginal Lecture Notes)

- Variant (vasospastic, Prinzmetal's) angina: This syndrome is a direct result of reduction in coronary flow, not an increase in myocardial oxygen demand
  - Channel blockers are effective in treating variant angina due to their effects on coronary dilatation rather than alterations in peripheral hemodynamics
  - Verapamil, nifedipine, nicardipine, bepridil, and diltiazem are all effective
- Exertional (exercise-induced) angina, angina of efffort: Usually due to fixed coronary vascular obstruction (surgical revascularization or angioplasty may be beneficial)
  - Therapeutic effect is due to increased coronary blood flow, and/or decreased myocardial oxygen consumption (secondary to decreased peripheral resistance, heart rate and contractility); the latter effect is thought to be more important
  - In some patients, channel blockers, particularly dihydropyridines, may aggravate anginal symptoms due to reflex increase in sympathetic tone, decreased coronary perfusion pressure, or coronary steal; not usually seen with verapamil or diltiazem
  - Concurrent therapy with beta-adrenergic blockers and/or nitrates can be more effective than individual agents alone:
    - Beta-adrenergic blockers suppress reflex tachycardia (and have negative dromotropic, chronotropic, and inotropic effects which reduce oxygen consumption)
    - Dihydropyridines will not enhance dromotropic effects of beta-blockers; concurrent treatment with verapamil or diltiazem is effective, but can lead to AV block, severe bradycardia, and decreased ventricular function
  - Nitrates cause venous dilation and reduce cardiac preload (channel blockers have no effect on venous return at normal doses)
- Unstable (preinfarction, crescendo) angina: Recurrent angina associated with minimal exertion; prolonged and frequent pain; ECG patterns indicate myocardial damage; coronary flow is severely restricted and vasospasm occurs in some patients; thought to be due to fissuring of atheroscelortic plaques and subsequent platelet aggregation
  - Usual therapy includes nitrates and beta-blockers which primarily act to reduce oxygen consumption and thereby control pain, and long-term use of aspirin to reduce possibility of thrombosis (inhibition of platelet aggregation)

- Ca^{++} channel blockers (especially verapamil) may be particularly effective if underlying mechanism involves vasospasm

Arrhythmias (also see Antiarrhthymics Lecture Notes)

- i.v. verapamil (followed by oral administration) is a drug of choice for interrupting and controlling paroxysysmal supraventricular tachycardias (i.e. -- originating from ectopic foci in atrial or junctional tissue)
  - Verapamil (i.v.) is useful in the immediate reduction of ventricular response in response to atrial fibrillation and flutter (except when associated with Wolff-Parkinson-White syndrome, a conduction abnormality)
    - **Contraindicated for atrial tachycardia caused by digitalis toxicity because of pharmacokinetic interactions that might lead to increased digoxin blood levels**
    - Can cause severe hypotension or ventricular fibrillation in patients with ventricular tachycardia (due to reflex increase in sympathetic tone); verapamil is rarely useful in treating ventricular arrhythmias
- Diltiazem and bepridil are currently being evaluated for use in treating arrhythmias and appear to be similar in efficacy to verapamil
- Bepridil may also be useful in treating ventricular arrhythmias because of its ability to also block Na^+ and K^+ channels (but can lead to the proarrhythmia, *torsades de pointes*)

Hypertension

- The calcium channel blockers are generally safe and are as effective as beta-adrenergic blockers or diuretics in the treatment of mild to moderate hypertension
- Calcium channel blockers are especially effective in treating low-renin hypertension (common in blacks and the elderly)
- Channel blockers are well-tolerated; minor side-effects include dizziness, headache, flushing, and edema and are most usually associated with the dihydropyridines
- **The use of L-type channel blockers should be avoided in patients with SA or AV nodal abnormalities or in patients with overt congestive heart failure**
- Efficacy can be enhanced by combination with other types of antihypertensives, but specific drug combinations should be avoided:
  - Diltiazem or verapamil with beta-adrenergic blockers (can lead to AV block, cardiac depression, bradycardia)
  - Ca^{++} -channel blockers with quinidine (reduced clearance of both drugs and potential pharmacodynamic effects at the SA and AV nodes)
  - Verapamil with digoxin (can increase levels of digoxin, possible AV block)

Less Common/Experimental Uses of Calcium Antagonists

- Subarachnoid hemorrhage (nimodipine)
- Treatment of migraine (nimodipine, nifedipine)
- Raynaud's phenomenon (nifedipine, diltiazem)
- Posthemorrhagic cerebral vasospasm (nimodipine)
- Inhibition of platelet aggregation (unknown mechanism)
- To slow development of atherosclerosis (mechanism unknown)
- Hypertrophic cardiomyopathy (nifedipine, verapamil)
- Postinfarct tissue preservation

[Home | About NetPharmacology | Lecture Notes | Test Questions | Drug List | Glossary | Figures]

Comments and suggestions to Donald K. Blumenthal, Ph.D.: dblument@alanine.pharm.utah.edu

Updated 11/10/98

British Journal of Pharmacology (1998) 124, 579–585
http://www.stockton-press.co.uk/bjp

T-type and L-type calcium channel blockers exert opposite effects on renin secretion and renin gene expression in conscious rats

[1,4]Charlotte Wagner, [2]Bernhard K. Krämer, [3]Markus Hinder, [1]Martin Kieninger & [1]Armin Kurtz

[1]Physiologisches Institut and [2]Klinik und Poliklinik für Innere Medizin II der Universität Regensburg, Germany and [3]Hoffmann-La Roche AG Grenzach, Germany

1 This study aimed to investigate and to compare the effects of pharmacological T-type calcium channel and of L-type calcium channel blockade on the renin system. To this end, male healthy Sprague-Dawley rats were treated with the T-channel blocker mibefradil or with the L-channel blocker amlodipine at doses of 5 mg kg^{-1}, 15 mg kg^{-1} and 45 mg kg^{-1} per day for four days and their effects on plasma renin activity (PRA) and kidney renin mRNA levels were determined.

2 Whilst amlodipine lowered basal systolic blood pressure at 5 mg kg^{-1}, mibefradil had no effect on basal blood pressure in the whole dose range examined. Amlodipine dose-dependently induced up to 7 fold elevation of PRA and renin mRNA levels. Mibefradil significantly lowered PRA and renin mRNA levels at 5 mg kg^{-1} and moderately increased both parameters at a dose of 45 mg kg^{-1}, when PRA and renin mRNA levels were increased by 100% and 30%, respectively. In primary cultures of renal juxtaglomerular cells neither amlodipine nor mibefradil (0.1–10 μM) changed renin secretion.

3 In rats unilateral renal artery clips (2K-1C) mibefradil and amlodipine at doses of 15 mg kg^{-1} day^{-1} were equally effective in lowering blood pressure. In contrast mibefradil (5 mg kg^{-1} and 15 mg kg^{-1} day^{-1}) significantly attenuated the rise of PRA and renin mRNA levels, whilst amlodipine (15 mg kg^{-1}) additionally elevated the rise of PRA and renin mRNA levels in response to renal artery clipping.

4 These findings suggest that T-type calcium channel blockers can inhibit renin secretion and renin gene expression *in vivo*, whilst L-type calcium channel blockers act as stimulators of the renin system. Since the inhibitory effect of T-type antagonists is apparent *in vivo* but not *in vitro*, one may infer that the effect on the renin system is indirect rather than directly mediated at the level of renal juxtaglomerular cells.

Keywords: Mibefradil; amlodipine; plasma renin activity; renin mRNA

Introduction

Calcium ions play a pivotal role in the contraction of vascular smooth muscle cells and consequently in vascular resistance. Calcium antagonists, in particular inhibitors of transmembrane calcium entry into vascular smooth muscle cells (VSMC), are therefore powerful drugs in the treatment of hypertension (Cauvin *et al.*, 1983; Hermsmeyer, 1991; Burges & Moisey, 1994). The most effective in this context have been proven to be inhibitors of L-type calcium channels (MacCarthy, 1987; Osswald *et al.*, 1990). A major side effect of L-type calcium antagonists in the treatment of hypertension is activation of the renin-angiotensin system (Churchill, 1987; Osswald *et al.*, 1990; Cappucio *et al.*, 1991; Schricker *et al.*, 1996a) which may, at least partly, counteract the hypotensive effect of calcium antagonists. The stimulation of the renin system by calcium channel antagonists is probably a multifactorial effect involving systemic effects such as a fall in blood pressure (Messing *et al.*, 1991; Bond & Boot, 1992) and activation of sympathetic outflow (Kailasam *et al.*, 1995) on the one hand, and more direct effects on the level of renal juxtaglomerular (JG) cells, which are the main site of renin gene expression and secretion. From these cells, renin is secreted by regulated exocytosis and evidence has been accumulated to indicate that exocytosis of renin is inhibited by a rise in the cytosolic calcium concentration (Hackenthal *et al.*, 1990).

Impairment of calcium entry into JG cells, which are metaplastically transformed vascular smooth muscle cells, is therefore expected to stimulate the secretion of renin. The pathways by which calcium enters renal renin-producing cells, have not clearly been identified but may involve store-regulated calcium influx directly in the juxtaglomerular region and L-type calcium channels in the more proximal parts of renal afferent arterioles (Scholz & Kurtz, 1996). Both pathways are inhibited by the second generation of dihydropyridines (Kass *et al.*, 1991), such as amlodipine.

Recently, a new generation of calcium channel blockers has been developed including mibefradil (Clozel *et al.*, 1990; Veniant *et al.*, 1991a,b) which selectively blocks T-type calcium channels (Mishira & Hermsmeyer, 1994). Mibefradil has been demonstrated to have similar potency to L-type calcium channels blockers in lowering blood pressure during hypertensive states (Hefti *et al.*, 1990; Veniant *et al.*, 1993; 1994). How T-type channel blockers influence the renin system, in particular renin secretion and renin gene expression, is not yet known.

We are interested therefore to compare the effects of an established L-type channel blocker, such as amlodipine, with a T-type channel blocker, such as mibefradil, on the renin system in conscious animals as well as in isolated juxtaglomerular cells. Our findings show that low dose mibefradil inhibits renin secretion and renin gene expression *in vivo* but not *in vitro*. At higher doses mibefradil induced a moderate stimulation of renin secretion and renin gene expression. However, this was substantially smaller than the respective stimulation induced by amlodipine.

[4]Author for correspondence at: Institut für Physiologie, Universität Regensburg, D-93040 Regensburg, Germany.

Methods

Animal experiments

Male Sprague-Dawley rats (220–250 g) kept in the local animal house were used for these studies. For blockade of calcium channels, the animals were treated either with the L-type calcium channel antagonist amlodipine or with the T-type calcium channel antagonist mibefradil (Ro 40-5967, Hoffmann-La Roche, Basel, Switzerland) at daily doses of 5 mg kg^{-1}, 15 mg kg^{-1} and 45 mg kg^{-1} for four days. The drugs were applied by gavage in the morning of each experimental day. In the control group, water was applied by gavage at the same time points.

In a second series of experiments, amlodipine (15 mg kg^{-1} day^{-1}) or mibefradil (5 mg kg^{-1} and 15 mg kg^{-1} day^{-1}) were given for four days to rats carrying a left renal artery clip from the third day of drug treatment. For this purpose animals were anaesthetized with methohexitone (50 mg kg^{-1}) and then the left kidney was exposed by an abdominal incision, and sterile silver clips (Degussa AG, Darmstadt, Germany) with an inner diameter of 0.2 mm were placed on the left renal arteries. The corresponding control group to this set of experiments consisted of rats also carrying a left renal artery clip but not treated with amlodipine or mibefradil. Animals were examined two days after insertion of the renal artery clips.

At the end of the experiment the animals were killed by decapitation and blood was collected from the carotid arteries, EDTA (5 mM) was added to the blood and haematocrit and PRA were determined. The kidneys were rapidly removed, weighed, cut into halves and rapidly frozen in liquid nitrogen. The organs were stored at −80°C until isolation of total RNA, which was extracted from one of the frozen halves of kidneys as described by Chomczynski & Sacchi (1987).

Determination of preprorenin mRNA by RNase protection assay

Renin mRNA was measured by RNase protection as described previously (Holmer *et al.*, 1994). A preprorenin cRNA probe containing 296 base pairs of exon I and III, generated from a pGEM-4 vector carrying a PstI-KpnI restriction fragment of a rat preprorenin cDNA (Burnham *et al.*, 1987) was generated by transcription with SP6 RNA polymerase (Amersham Int., Amersham, U.K.). Transcripts were routinely labelled with [^{32}P]-GTP (400 Ci $mmol^{-1}$; Amersham International) and purified on a Sephadex G50 spin column. For hybridization total kidney RNA was dissolved in a buffer containing 80% v/v formamide, 40 mM piperazine-N,N'-*bis*(2-ethane sulphonic acid), 400 mM NaCl, 1 mM EDTA (pH 8); 20 μg of kidney RNA were hybridized in a total volume of 50 μl at 60°C for 12 h with 5×10^5 c.p.m. radiolabelled renin probe. RNase digestion with RNase A and T1 was carried out at room temperature for 30 min and terminated by incubation with proteinase K (0.1 mg ml^{-1}) and SDS (0.4% w/v) at 37°C for 30 min.

Protected preprorenin mRNA fragments were purified by phenol/chloroform extraction, ethanol precipitation and subsequent electrophoresis on a denaturing 8% w/v polyacrylamide gel. After autoradiography of the dried gel at −80°C for one day bands representing protected renin mRNA fragments were excised from the gel and radioactivity was counted with a liquid scintillation counter (1500 Tri-CarbTm, Packard Instrument Company, Downers Grove, Illinois, U.S.A.).

Determination of glyceraldehyde-3-phosphate dehydrogenase-mRNA (GAPDH)

The presence of rat GAPDH-mRNA in total RNA was measured by RNase protection assay. A GAPDH-cRNA probe containing a fragment of 342 bp of rat GAPDH-cDNA (Tso *et al.*, 1985) was generated from a pGEM-4Z vector (Pharmacia) after linearization with *Hind*III and following transcription with SP6-polymerase. GAPDH-mRNA was used as a standard RNA controlling the quality of the RNA preparation. Total RNA (1 μg) was hybridized under the conditions described for the determination of preprorenin mRNA.

Measurement of heart rate and blood pressure

Heart rate and systolic blood pressure was measured by the tail cuff method with a BP recorder 8005 (Rhema, Hofheim, Germany) at 08 h 00 min and 16 h 00 min of each experimental day.

Determination of plasma renin activity

PRA was determined by incubation of the plasma samples at 37°C (pH 6) for 1 h and measurement of the angiotensin I, generated using a commercially available radioimmunoassay kit for angiotensin I (Sorin Biomedica, Düsseldorf, Germany).

Experiments with cultured juxtaglomerular cells

Mouse juxtaglomerular cells were isolated as described previously (Della Bruna *et al.*, 1992). In order to prepare 3 ml of final cell suspension, one C57BL6 mouse (4–6 weeks old) that had free access to normal food and water (Altromin, Lage, Germany) was killed by decapitation. The kidneys were removed, decapsulated and minced with a scalpel blade. The minced tissue was incubated with gentle stirring in buffer 1 (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM glucose, 20 mM sucrose, 10 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.4) supplemented with 0.25% w/v trypsin (Sigma, Deisenhofen, Germany) and 0.1% collagenase (0.5 u mg^{-1}, type A, Boehringer Mannheim, Germany) at 37°C for 70 min. After enzymatic dissociation, the tissue was sieved over a 22 μm screen. Single cells passing the screen were collected, washed and resuspended in 4 ml of buffer 1 and then further separated using Percoll (Pharmacia, Uppsala, Sweden) density gradients. The cell suspension obtained was added to two tubes each containing 30 ml of 30% v/v isoosmotic Percoll in buffer 1. After 25 min of centrifugation at 4°C and 27,000 *g*, four cell layers with different specific renin activity were obtained. The cellular layer (density = 1.07 g ml^{-1}) with the highest specific renin activity was used for cell culture.

These cells were washed in buffer 1 and resuspended in RPMI-1640 medium (Biochrom, Berlin, Germany) containing 0.66 u ml^{-1} insulin, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2% v/v foetal calf serum (FCS). The cultures were distributed in 100 μl aliquots into 96-well plates. The cultures were incubated at 37°C in humidified atmosphere containing 5% CO_2 in air.

After 24 h of primary culture, the culture medium was removed, and the cultures were washed once with 100 μl RPMI-1640 medium containing 2% v/v FCS. Then 100 μl of fresh and prewarmed culture medium with the chemicals to be tested were added.

Experiments on renin secretion were performed for the next 20 h of incubation. At the end of experiments,

supernatants were collected and centrifuged at 1000 g at room temperature to remove cellular debris. The supernatants were then stored at −20°C until assayed for renin activity. Cells were lysed by adding to each culture well of 100 µl PBS containing 0.1% Triton X-100 and shaking for 45 min at room temperature. The lysed cells were stored at −20°C until further processing.

Renin secretion rates were estimated from the appearance rate of renin in the culture medium. To minimize differences between different cell culture preparations, renin secretion rates were calculated as fractional release of total renin found at the end of primary culture (i.e. renin activity released/(renin activity released + renin activity remaining in the cells)).

Statistics

Significance levels were calculated with the ANOVA test. A value of $P<0.05$ was considered significant.

Results

Effect of drugs on systolic blood pressure

Animals were treated with the drugs for 4 days. At that time systolic blood pressure was significantly lowered in animals receiving amlodipine but not in animals receiving mibefradil (Figure 1). The lowest dose of amlodipine used in this study (5 mg kg^{-1} day^{-1}) lowered systolic blood pressure by about 20 mmHg.

Effect of drugs on heart rate

The lowest dose of amlodipine used (5 mg kg^{-1} day^{-1}) increased heart rate by about 20 beats min^{-1} (Figure 2). The increase in heart rate was not further enhanced by higher doses of amlodipine but the scatter of values became smaller with high doses of amlodipine. In contrast, mibefradil exerted a clear dose-dependent inhibitory effect on heart rate (Figure 2).

Effect of drugs on plasma renin activity

PRA was determined in the animals as an indirect measure of renal renin secretion. Figure 3 shows that amlodipine caused a dose-dependent rise of PRA which was elevated by 500% above normal in animals treated with the highest dose of 45 mg kg^{-1} day^{-1}. Mibefradil treatment exerted a dual effect on PRA values. At a dose of 5 mg kg^{-1} day^{-1}, PRA was significantly lowered. At a dose of 15 mg kg^{-1} day^{-1} PRA values were not different from controls. At 45 mg kg^{-1} day^{-1} mibefradil PRA values were significantly increased by about 200% (Figure 3b).

Effect of drugs on renal renin mRNA levels

Similar to its effect on PRA, amlodipine also dose-dependently increased renin mRNA levels by about 700% with the highest dose of 45 mg kg^{-1} day^{-1} (Figure 4a). Mibefradil at a dose of 5 mg kg^{-1} day^{-1} moderately but significantly lowered renin mRNA levels. At the highest dose of 45 mg kg^{-1} day^{-1}, renin mRNA levels were increased by about 30% versus control animals without reaching a level of statistical significance ($P>0.05$) (Figure 4b).

Effects of drugs on renin secretion from isolated juxtaglomerular cells

To test for a possible direct effect of amlodipine and of mibefradil at the level of the renal juxtaglomerular cells, the effects of both drugs were examined in primary cultures of mouse juxtaglomerular cells. As shown in Figure 5 neither drug affected basal renin secretion from cultured cells, which

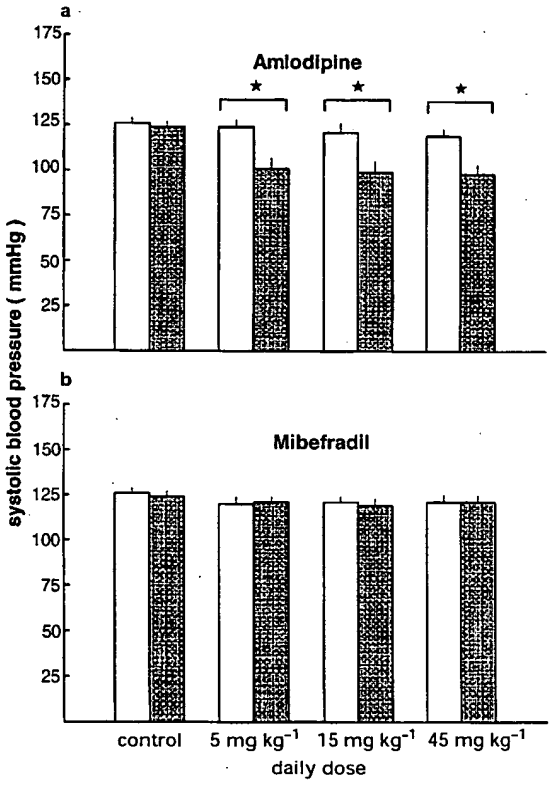


Figure 1 Dose-response effect of treatment with amlodipine (a) and mibefradil (b) for 4 days on basal systolic blood presure in conscious rats. Data show values of systolic blood pressure measurements (mmHg) at the beginning (open columns) and at the end (solid columns) of the experiments. Data are mean±s.e.mean, $n=6$; $^*P<0.05$.

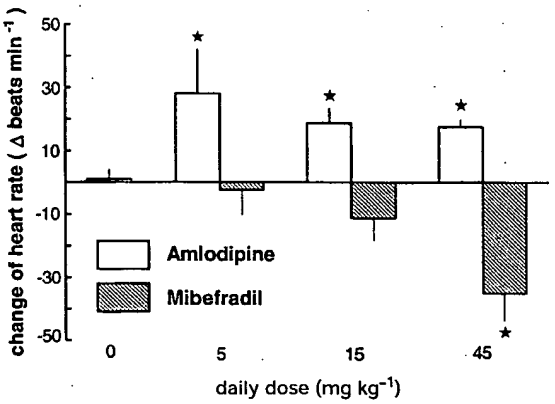


Figure 2 Dose-response effect of treatment with amlodipine and mibefradil for 4 days on heart rate in the conscious rat. Data show changes in heart rate (beats min^{-1}) and are means±s.e.mean, $n=6$; $^*P<0.05$.

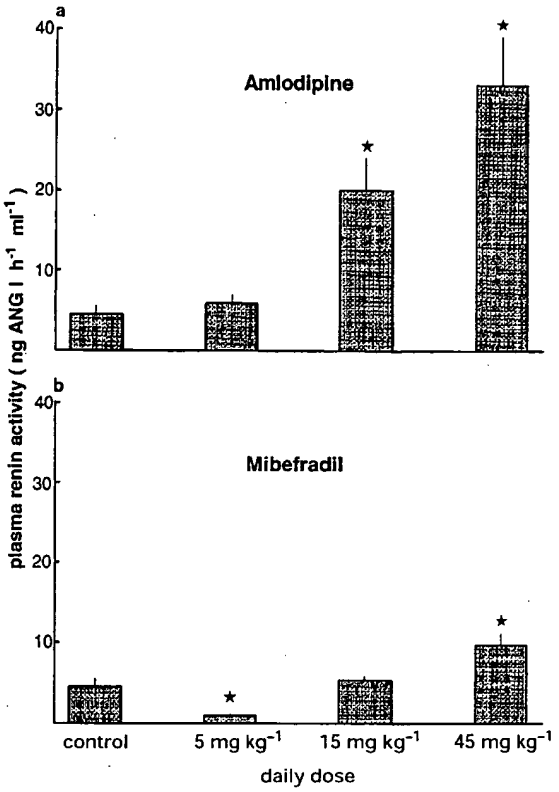


Figure 3 Dose-response effect of treatment with amlodipine (a) and mibefradil (b) for 4 days on PRA. Data show changes in PRA (ng angiotensin I h^{-1} ml^{-1}) and are means ± s.e.mean, $n=6$; $^*P<0.05$.

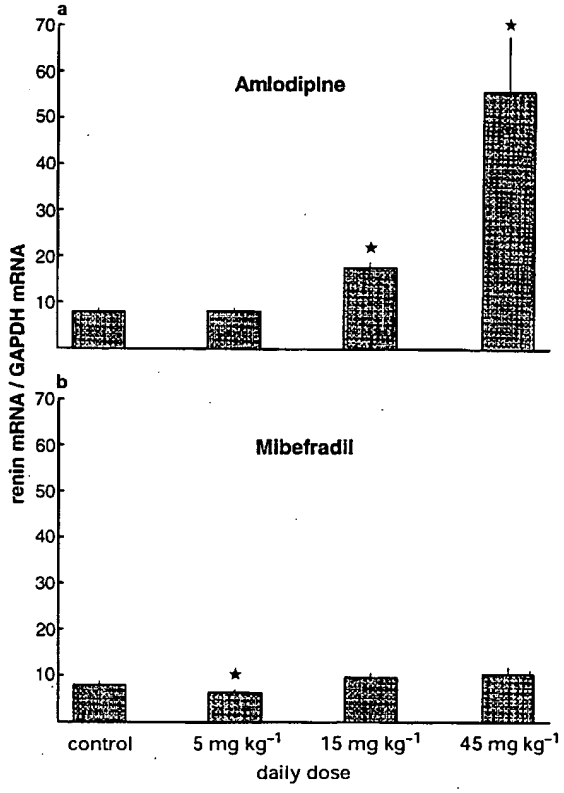


Figure 4 Dose-response effect of treatment with amlodipine (a) and mibefradil (b) for 4 days on renin mRNA. Data show changes in renal renin mRNA levels and are means ± s.e.mean, $n=6$, $^*P<0.05$.

spontaneously released about 18% of stored active renin within 20 h of culture. Renin secretion was significantly stimulated by a lowering of the extracellular calcium concentration and by activation of adenylate cyclase with forskolin (Figure 5).

Effect of drugs on plasma renin activity, renin mRNA levels and blood pressure in 2K-1C rats

To examine the effects of calcium antagonists on the stimulated renin system, amlodipine and mibefradil were administered to rats with unilateral renal artery stenosis. In animals without drug treatment, PRA increased about four fold two days after clipping (Figure 6). At that time, renin mRNA was increased about five fold in the clipped kidneys and had decreased to about 30% of the normal value in the intact contralateral kidney (Figure 7). In animals pretreated with amlodipine (15 mg kg^{-1} day^{-1}) PRA increased about 10 fold two days after clipping (Figure 6). Renin mRNA in the clipped kidney had increased about six fold above control (Figure 7a). Renin mRNA levels in the contralateral intact kidney were not different from the values found in normal untreated animals (Figure 7b). In animals pretreated with mibefradil (5 mg kg^{-1} day^{-1}) the increase in PRA, as well as the increase in renin mRNA in the stenosed kidneys two days after clipping, were greatly reduced (Figure 7a), whilst renin mRNA in the contralateral intact kidney fell significantly below the value found in normal kidneys (Figure 7b). At a dose of 15 mg kg^{-1} day^{-1} mibefradil still attenuated the

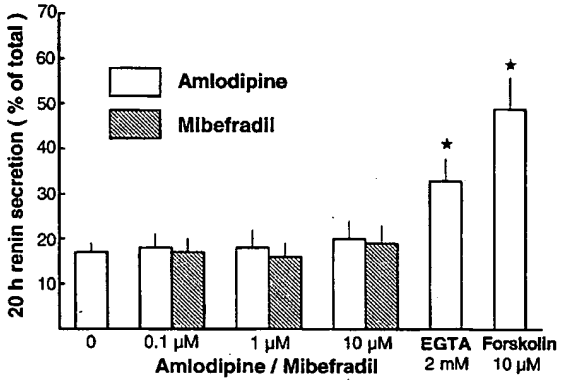


Figure 5 Renin secretion from mouse cultured JG cells under basal conditions (control) and in the presence of amlodipine (0.1–10 µM), mibefradil (0.1–10 µM), EGTA (2 mM) and forskolin (10 µM). Data are means ± s.e.mean of different cell preparations, $n=5$. Total renin activity in the different preparations was 48 ± 12 µg angiotensin I h^{-1} mg^{-1} cellular protein. Each experiment represents the mean of 4 replicate culture wells. $^*P<0.05$.

increase of PRA and of renin mRNA levels in response to renal artery clipping.

Unilateral renal artery clipping increased systolic blood pressure by about 30 mmHg (Figure 8). In animals receiving either amlodipine or mibefradil (15 mg kg^{-1} day^{-1}) together

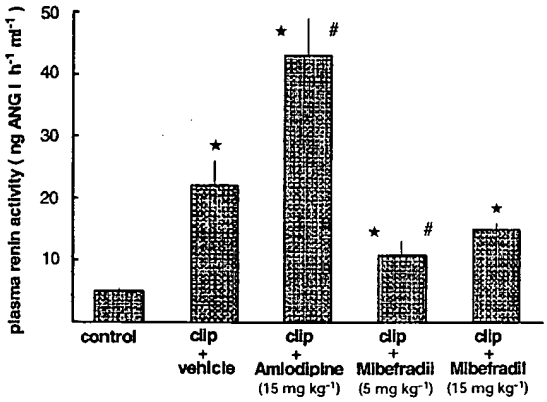


Figure 6 Effect of treatment with amlodipine and mibefradil for 4 days on PRA in 2K-1C rats. Data show changes in PRA (ng angiotensin h^{-1} mg^{-1}) and are means ± s.e.mean, $n=6$; $^*P<0.05$ vs control; $\#P<0.05$ vs clipped, vehicle-treated animals.

with a renal artery clip, systolic blood pressure was not changed relative to the starting values (Figure 8). At a dose of 5 mg kg^{-1} day^{-1} mibefradil did not prevent the increase in blood pressure in response to renal artery clipping (Figure 8).

Discussion

This study aimed to compare the effects of the L-type calcium channel blocker amlodipine and the T-type calcium channel blocker mibefradil on renin secretion and renin gene expression. In accordance with previous results (Schricker *et al.*, 1996a) we found that amlodipine in the concentration range between 5 mg kg^{-1} day^{-1} and 45 mg kg^{-1} day^{-1} lowered systolic blood pressure at the lowest dose examined in this study. Mibefradil on the other hand exerted no effect on basal systolic blood pressure over the whole dose range examined. The inability of mibefradil to lower basal blood pressure seems to be at odds with previous studies showing a reduction of blood pressure in normal animals (Hefti *et al.*, 1990). The majority of these studies used i.v. bolus injections of mibefradil and the experimental protocol is therefore not comparable with this study (e.g. Veniant *et al.*, 1991a). One study showed that mibefradil administered by daily gavage significantly lowered systolic blood pressure after six days of treatment (Schmitt *et al.*, 1995). However, in that study basal blood pressure of rats was substantially higher than in the present experiments. Thus mibefradil lowered systolic blood pressure to values between 125 to 130 mmHg which were considered as normal blood pressure values in this study, whilst amlodipine lowered blood pressure to values around 100 mmHg what was also found in this study. It appears that orally administered mibefradil has little effect on normal blood pressure values and is primarily effective during hypertensive states (Boulanger *et al.*, 1994). This conclusion is supported by the observation that mibefradil prevented the rise of blood pressure in response to renal artery clipping.

In contrast to its lack of effect on basal blood pressure, mibefradil dose-dependently lowered the heart rate, which is in good agreement with previous observations (Veniant *et al.*, 1991a). The mechanism of the negative chronotropic effect of mibefradil is not yet understood but could involve direct effects on the sinoatrial node as well as indirect effects such as a decrease in sympathetic activity. In contrast to mibefradil and

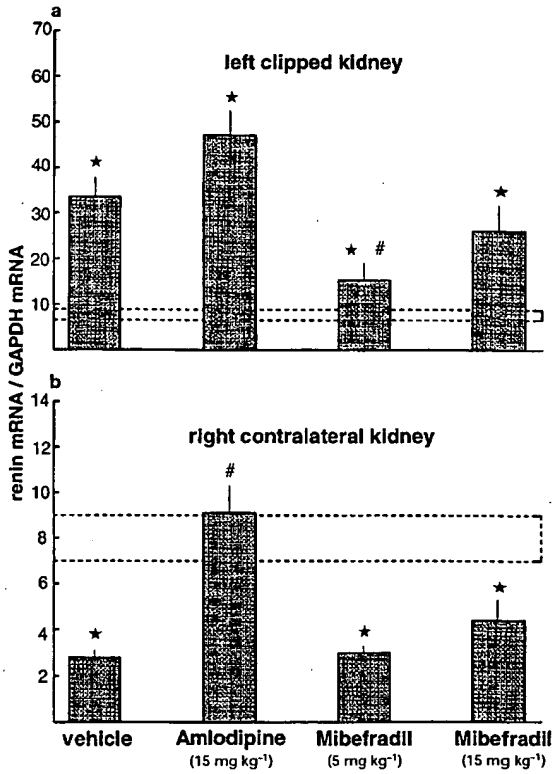


Figure 7 Effect of treatment with amlodipine and mibefradil for 4 days on renin mRNA in clipped (a) and contralateral (b) kidneys of 2K-1C rats. Data show changes in renal renin mRNA levels and are means ± s.e.mean, $n=6$; $^*P<0.05$ vs control; $\#P<0.05$ vs clipped, vehicle-treated animals.

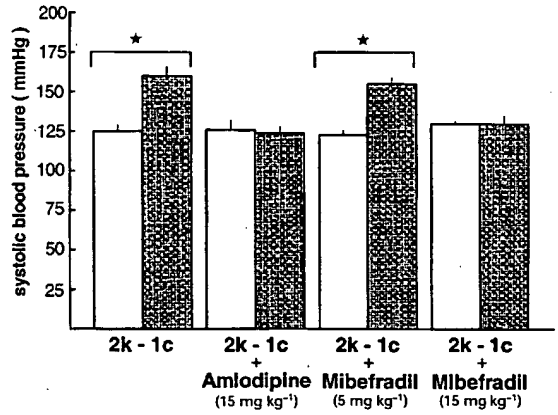


Figure 8 Effect of treatment with amlodipine and of mibefradil for 4 days on systolic blood pressure in conscious 2K-1C rats. Data show values of blood pressure measurements (mmHg) at the beginning (open columns) and at the end (solid columns) of the experiments. Data are means ± s.e.mean, $n=6$; $^*P<0.05$.

in accordance with previous observations (Veniant *et al.*, 1991a), amlodipine significantly increased heart rate probably due to an activation of sympathetic nerves. Amlodipine (45 mg kg^{-1}) dose-dependently increased PRA up to six fold and renin mRNA levels up to seven fold.

Mibefradil exerted a dual effect on basal renin secretion and renin gene expression *in vivo*. At 5 mg kg^{-1} day^{-1}, mibefradil clearly reduced PRA and also moderately but significantly lowered renin mRNA levels. Increasing the daily dose of mibefradil to 45 mg kg^{-1} caused a moderate increase in PRA and renin mRNA levels. However, at each dose the stimulant effect of mibefradil of renin was far smaller than that of amlodipine. In isolated renal juxtaglomerular cells neither amlodipine nor mibefradil stimulated renin secretion, suggesting that the effects of both drugs *in vivo* on the renin system were indirect. In the concentration range of 0.1 – 10 μM amlodipine and mibefradil would be expected to block L-type and T-type calcium channels respectively (Mishira & Hermsmeyer, 1994; Koidl *et al.*, 1997). It was notable that lowering extracellular calcium concentration stimulated renin secretion from cultured juxtaglomerular cells, which supports the conclusion that neither L-type nor T-type calcium channels are effectively involved in the control of renin secretion at the cellular level.

The inhibitory effect of low mibefradil doses on the renin system was exerted in situations where the renal renin system was stimulated. Such a stimulation was induced by unilateral renal hypoperfusion due to placement of a renal artery clip. Two days after placement of the renal artery clips PRA was increased about 4 fold and renin mRNA was increased about 5 fold in the clipped kidney, whilst renin mRNA was markedly suppressed in the contralateral, normal kidney. Pretreatment of animals with amlodipine (15 mg kg^{-1} day^{-1}) before renal artery clipping additionally elevated the rise of PRA and of renin mRNA. Mibefradil (5 mg kg^{-1} and 15 mg kg^{-1}), on the other hand, led to a clear attenuation of the rise of PRA and of renin mRNA in response to renal artery clipping.

By which indirect pathways amlodipine may stimulate and mibefradil may inhibit the renin system *in vivo* remains unclear. Apart from blood pressure, important determinants for the activity of the renin system *in vivo* comprise the salt load of the body (Holmer *et al.*, 1993; Schricker *et al.*, 1996b), sympathetic outflow (Holmer *et al.*, 1994), a yet unidentified macula densa signal (Modena *et al.*, 1993) as well as local factors such as intrarenal formation of angiotensin II, prostaglandins or nitric oxide (Johns *et al.*, 1990; Schricker *et al.*, 1995a,b).

Although our study cannot clearly distinguish between these various possibilities an obvious candidate could be sympathetic nerve activity. The changes of heart rate observed could be interpreted as a stimulant and an inhibition of sympathetic nerve activity by amlodipine and mibefradil, respectively. Thus it will be of interest to characterize the role of the sympathetic nervous system in the effects of calcium channel blockers on the renin system *in vivo*.

The expert technical assistance provided by K.H. Götz and M. Hamann is gratefully acknowledged.

References

BOND, A. & BOOT, J.R. (1992). The effect of calcium channel modulators on blood pressure and pressor responses to noradrenaline in the guinea pig. *Eur. J. Pharmacol.*, **218**, 179–181.

BOULANGER, C.M., DESTA, B., CLOZEL, J.P. & VANHOUTTE, P.M. (1994). Chronic treatment with the Ca^{2+} channel inhibitor Ro 40-5967 potentiates endothelium-dependent relaxations in the aorta of the hypertensive salt sensitive Dahl rat. *Blood Pressure*, **3**, 193–196.

BURGES, R. & MOISEY, D. (1994). Unique pharmacologic properties of amlodipine. *Am. J. Cardiol.*, **73**, 2A–9A.

BURNHAM, C.E., HAWELY-JOHNSON, C.L., FRANK, B.M. & LYNCH, K.B. (1987). Molecular cloning of rat renin cDNA and its gene. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 5605–5609.

CAPPUCIO, F.P., MARKANDU, N.D., SAGNELLA, G.A., SINGER, D.R., BUCKLEY, M.G., MILLER, M.A. & MACGREGOR, G.A. (1991). Effects of amlodipine on urinary sodium excretion, renin-angiotensin-aldosterone system, atrial peptide and blood pressure in essential hypertension. *J. Hum. Hypertens.*, **5**, 115–119.

CAUVIN, C., LOUTZENHISER, R. & VAN BREMEN, C. (1983). Mechanism of calcium antagonist-induced vasodilation. *Annu. Rev. Pharmacol. Toxicol.*, **23**, 373–396.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single step method of RNA isolation by acid guanidinium thiocaynate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.

CHURCHILL, P.C. (1987). Calcium channel antagonists and renin release. *Am. J. Nephrol.*, **7**, 32–38.

CLOZEL, J.P., VENIANT, M. & OSTERREIEDER, W. (1990). The structurally novel Ca^{2+} channel blocker Ro 40-5967, which binds to the [^{3}H] desmethoxyverapamil in normal and failing rat hearts. *Cardiovasc. Drugs Ther.*, **4**, 731–736.

DELLA BRUNA, R., PINET, F., CORVOL, P. & KURTZ, A. (1992). Regulation of renin secretion and renin synthesis by second messengers in isolated mouse juxtaglomerular cells. *Cell Physiol. Biochem.*, **1**, 98–110.

HACKENTHAL, E., PAUL, M., GANTEN, D. & TAUGNER, R. (1990). Morphology, physiology and molecular biology of renin secretion. *Physiol. Rev.*, **70**, 1067–1116.

HEFTI, F., CLOZEL, J.-P. & OSTERRIEDER, W. (1990). Antihypertensive properties of a novel calcium antagonist (1S,2S)-2-[2-[[3-(2-benzimidazolyl)propyl]methylamino]-ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isoprophyl-2-naphthyl methoxyacetate dihydrochloride in rat models of hypertension. *Drug. Res.*, **40**, 417–421.

HERMSMEYER, K. (1991). Differences of calcium channels in vascular muscle in hypertension. *Am. J. Hypertens*, **4**, 4125–4159.

HOLMER, S., ECKARDT, K.U., LEHIR, M., SCHRICKLER, K., RIEGGER, G. & KURTZ, A. (1993). Influence of dietary NaCl intake on renin gene expression in the kidneys and adrenal glands of rats. *Pflügers Arch.*, **425**,

HOLMER, S., RINNE, B., ECKARDT, K.U., LE HIR M., SCHRICKER, K., KAISSLING, B., RIEGGER, G. & KURTZ, A. (1994). Role of renal nerves for the expression of renin in adult rats kidney. *Am. J. Physiol.*, **266**, F738–F745.

JOHNS, D.W., PEACH, M.J., GOMEZ, R.A., INAGAMI, T. & CAREY, R.M. (1990). Angiotensin II regulates renin gene expression. *Am. J. Physiol.*, **259**, F882–F887.

KAILASAM, M.T., PARMER, R.J., CERVENKA, J.H., WU, R.A., ZIEGLER, M.G., KENNEDY, B.P., ADEGBILE, I.A. & O'CONNOR, D.T. (1995). Divergent effects of dihydropyridine and phenylalkylamine calcium channel antagonist classes on autonomic function in human hypertension. *Hypertension*, **26**, 143–149.

KASS, R.S., ARENA, J.P. & CHIN, S. (1991). Block of L-type calcium channels by charged dihydropyridines. Sensitivity to side of application and calcium. *J. Gen. Physiol.*, **98**, 63–75.

KOIDL, B., MIYAWAKI, N. & TRITTHART, H.A. (1997). A novel benzothiazine Ca^{2+} channel antagonist, semotiadil, inhibits cardiac L-Type Ca2+ currents. *Eur. J. Pharmacol.*, **322**, 243–247.

MACCARTHY, E.P. (1987). Dihydropyridines and beta-adrenoceptor antagonists as combination treatment in hypertension. *J. Hypertens. Suppl.*, **4**, 133–137.

MESSING, M., VAN ESSEN, H., SMITH, T.L., SMITS, J.F. & STRUYKER-BOUDIN, H.A. (1991). Microvascular actions of calcium channel antagonists. *Eur. J. Pharmacol.*, **198**, 189–195.

MISHIRA, S.K. & HERMSMEYER, K. (1994). Selective inhibition of T-type Ca2+ channels by Ro 40-5967. *Circ. Res.*, **75**, 144–148.

MODENA, B., HOLMER, S., ECKARDT, K.U., SCHRICKER, K., RIEGGER, G., KAISSLING, B. & KURTZ, A. (1993). Furosemide stimulates renin expression in the kidneys of salt-supplemented rats. *Pflügers Arch.*, **424**, 403–409.

OSSWALD, H., WEINHEIMER, G. & KAPP, J.F. (1990). Renal actions of calcium channel antagonists. *J. Neural. Transm. Suppl.*, **31**, 39–53.

SCHOLZ, H. & KURTZ, A. (1996). Differential regulation of cytosolic calcium between afferent arteriolar smooth muscle cells from mouse kidney. *Pflügers Arch.*, **431**, 46–51.

SCHMITT, R., CLOZEL, J.P., IBERG, N. & BÜHLER, F.R. (1995). Mibefradil prevents neointima formation after vascular injury in rats. *Arterioscl. Thromb. Vasc. Biol.*, **15**, 1161–1165.

SCHRICKER, K., HAMANN, M. & KURTZ, A. (1995a). Prostaglandins are involved in the stimulation of renin gene expression in 2 kidney-1 clip rats. *Pflügers Arch.*, **430**, 188–194.

SCHRICKER, K., HEGYI, I., HAMANN, M., KAISSLING, B. & KURTZ, A. (1995b). Tonic stimulation of renin gene expression by nitric oxide is counteracted by tonic inhibition through angiotensin II. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 8006–8010.

SCHRICKER, K., HAMANN, M., MACHER, A., KRÄMER, B.K., KAISSLING, B. & KURTZ, A. (1996a). Effect of amlodipine on renin secretion and renin gene expression in rats. *Br. J. Pharmacol.*, **119**, 744–750.

SCHRICKER, K., PÖTZL, B., HAMANN, M. & KURTZ, A. (1996b). Coordinate changes of renin and brain-type nitric-oxide-synthase (b-NOS) mRNA levels in rat kidneys. *Pflügers Arch.*, **432**, 394–400.

TSO, J.Y., SUN, X.H., KAO, T.H., REECE, K.S. & WU, R. (1985). Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res.*, **13**, 2485–2502.

VENIANT, M., CLOZEL, J.P., HESS, P. & WOLFGANG, R. (1991a). Hemodynamic profile of Ro 40-5967 in conscious rats: comparison with diltiazem, verapamil and amlodipine. *J. Cardiovasc. Pharmacol.*, **18**, S55–S58.

VENIANT, M., CLOZEL, J.P., HESS, P. & WOLFGANG, R. (1991b). Ro 40-5967, in contrast to diltiazem, does not reduce left ventricular contractility in rats with chronic myocardial infarction. *J. Cardiovasc. Pharmacol.*, **17**, 277–284.

VENIANT, M., CLOZEL, J-P., HEUDES, D., BANKEN, L. & MENARD, J. (1993). Effects of Ro 40-5967, a new calcium angatonist, and enalapril on cardiac remodeling in renal hypertensive rats. *J. Cardiovasc. Pharmacol.*, **21**, 544–551.

VENIANT, M., HEUDES, D., CLOZEL, J.P., BRUNEVAL, P. & MENARD, J. (1994). Calcium blockade versus ACE inhibition in clipped and unclipped kidneys of 2K-1C rats. *Kidney Int.*, **46**, 421–429.

(Received April 30, 1997
Revised February 23, 1998
Accepted February 26, 1998)